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Molecular genetic assessment of chicken macrophage innate immunity: toll-like receptors,  
mechanisms of action, and kinetic transcriptome profile

By

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

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Ames, Iowa

2010

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*To my family and Sue Lamont*

*Ailem ve Sue Lamont'a*

*Sevgilerimle...*

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## Abstract

Understanding the genetic regulation of host response governing disease resistance mechanisms is of primary importance for improving animal health and food safety. Many of the biological characteristics of the chicken make it an ideal organism for studies in immunology, evolution, agriculture, medicine and comparative functional analyses. Different cell lines or primary cells from the immune system generate different immune responses even though they are induced with the same stimulant, suggesting that it is important to dissect the immune system to understand the mechanisms that are shaping the host response. Therefore, we used chicken macrophages as a model to study these mechanisms. This dissertation involves a comprehensive set of experimental and bioinformatic studies designed to deepen the current knowledge of chicken macrophage innate immune responses. In the first study, we determined the role of Toll-like receptor 15 (TLR15) and its downstream effector responses in the chicken macrophage HD11 cell line. TLR15 is an avian-specific pattern recognition receptor (PRR) of unknown specificity that is structurally different from other TLRs in the arrangement of the leucine rich region (LRR). TLR15, in collaboration with, TLR2 and TLR21 can respond to CpG oligonucleotides (ODN) and is able to rapidly distinguish different types of CpG-ODNs, as does mammalian TLR9. Using RNA interference technology, we demonstrated that the MyD88 adaptor molecule is required to have upregulation of Interleukin 1 beta (IL1B) after induction of TLR15 gene expression with CpG-ODNs. In a second, the effect of *Salmonella typhimurium*-798 (ST-798)-derived endotoxin on kinetic mRNA profiles of chicken macrophages HD11 cells at 1, 2, 4 and 8 hours post-stimulation (hps) was tested using *Affymetrix* GeneChip chicken genome arrays

containing 38535 probesets. By comparison with non-stimulated cells, the greatest gene expression response was observed at 4 hps. Comparative analysis of gene networks from the microarray data indicated that 10% of the differentially expressed genes were involved in the response to *Salmonella* endotoxin. Additionally, this work demonstrated a consistent signature set of four up-regulated genes at all tested time points: IL1BB, IL8, NFKBIA, and CCL4. The differentially expressed genes obtained from microarray analysis were categorized by function with emphasis on inflammatory response. The examination of chicken macrophage immune response on a global scale and the investigation of regulatory mechanisms of innate response are the main objectives of this dissertation. Our findings hold great potential to expand possibilities for production of better pharmaceuticals, and identification of beneficial genetic selection markers, by defining immune response pathways.

# Chapter 1. General Introduction

## Organization of Dissertation

This dissertation is organized in an alternative format that consists of four chapters. Chapter 1 is the literature review, chapter 2 is the manuscript accepted for publication in *Developmental and Comparative Immunology* and chapter 3 is published in *BMC Genomics* and chapter 4 contains the discussion, general conclusions and future perspectives. Figures and tables are included in each manuscript following the reference section. Each chapter is followed by reference citations that are formatted consistently, regardless of the scientific journals to which individual manuscripts were submitted for publication.

## Introduction

The chicken has been studied as a model in developmental biology for more than 100 years (Stern, 2005). Availability of the chicken genome sequence has emphasized the importance of the chicken as a model organism (Edwards *et al.*, 2005). The availability of genomic sequence information has led to a detailed analysis and characterization of many genes in the chicken, including immune response related genes such as cytokines and chemokines (Kaiser *et al.*, 2005).

Macrophages are primary cells that are involved in the recognition of bacterial lipopolysaccharide (LPS) (Moore *et al.*, 1976). Upon stimulation, macrophages undergo an activation process involving an increase in size and mobility, increased phagocytic and bactericidal activity and up-regulation of several cell surface markers (Bliss *et al.*, 2005).

Macrophages, as important mediators of host response to infections, secrete cytokines and chemokines that regulate migration of white blood cells to the site of the infection and initiate antimicrobial mechanisms to eradicate pathogens (Hong *et al.*, 2006).

Most classes of vertebrates are relatively resistant to LPS. Birds are susceptible in embryonic development (Finkelstein, 1964), as opposed to mammals which are sensitive at post-developmental stages (Berczi *et al.*, 1966). The mode of cellular activation by LPS must be specific, raising the question why it elicits responses from some cells and not from others in a given organism (Beutler and Rietschel, 2003). As in mammalian cells, macrophages in chickens play substantial roles in both innate and adaptive immunity. The primary requirement for any immune system is the ability to recognize the invasion of pathogens in tissues and to initiate an immune response. Pathogens are sensed by an array of specialized pattern recognition receptors (PRR), most of which are common and conserved in higher order species, including chicken, in spite of the divergence of birds and mammals 310 million years ago (Burt, 2005). Investigating PRRs such as Toll-like receptors (TLRs) that are in the conserved regions of chromosomes and examining the kinetics of LPS response might allow an exploration of the similarity of mammalian and avian immune systems.

One of the important fields that has emerged since the identification of innate immunity is the topic of TLRs. Once TLRs come in contact with stimuli, these receptors then activate a whole cascade of events, resulting in cytokine and chemokine production to recruit inflammatory cells that are responsible for eradicating pathogens. Numbers and members of TLRs vary in different species. There are 10 TLRs in human, 12 in mice (Akira and Takeda, 2004) and 10 TLRs in chicken (Temperley *et al.*, 2008). A functional gene for TLR11 has

only been found in mice; the TLR7, 8, and 9 subfamily is represented by only TLR7 in chickens. The first TLR to be discovered was TLR4, which is absolutely essential for the recognition of endotoxin (Medzhitov *et al.*, 1997). TLRs are variably expressed by antigen presenting cells (APC) such as macrophages and dendritic cells and, more recently, intestinal epithelial cells (Cario and Podolsky, 2000) and even T cells have been reported to express TLRs (MacLeod and Wetzler, 2007). In addition to lipid-based structures such as endotoxin, which is recognized by TLR4, there are TLRs that specifically recognize other pathogen-associated components such as nucleic acids (CpG-ODN/TLR9, ssRNA and dsRNA/TLR3), lipoproteins from gram positive bacteria (TLR2), and flagella (TLR5). The events initiated inside the cells when a given TLR is induced have been the focus of much investigation. Our studies will generate new insights into how TLRs work at the molecular level and more accurate interpretation of the signals initiated as a result of infectious and/or inflammatory diseases.

Microarrays have become a standard tool in molecular genetics because they provide information on expression levels for thousands of genes simultaneously (Baum *et al.*, 2003). The differentially expressed genes can be investigated with different pathway analysis bioinformatics tools to connect with known biological pathways by using public sources. The integration of the differentially expressed genes into known biological pathways is an ideal tool to dissect the complexity of gene expression (Jimenez-Marin *et al.*, 2009).

This dissertation aims to address questions regarding the chicken host response to specific stimulators and the mechanism of action shaping this host response by utilizing



contemporary technologies including RNA interference, real-time QPCR and microarray analysis to define cellular responses.

## **Literature Review**

### **Innate and adaptive immunity**

#### **General concepts**

Living organisms are constantly exposed to foreign substances including food, microorganisms, unnecessary metabolites, even transformed self molecules (Cooper *et al.*, 2001), hence generating a need to distinguish dangerous non-self from self entities, especially invasive microorganisms. All vertebrates have evolved two classes of defense against invading pathogens: innate and adaptive immunity (Beck and Habicht, 1996). The first line of defense against pathogen invasion is mediated by the innate immune system. This system requires microbe recognition receptors including complement receptors, lectins, and Toll-like receptors (TLRs) (Medzhitov *et al.*, 1997). The innate immune system responds to infections with rapid kinetics, but it lacks memory capabilities. The promptness of the response is an outcome of the availability of innate and natural immune cells that express receptors before exposure to the stimulants (Kawai and Akira, 2010). Although these receptors are mostly expressed by monocytes/macrophages and dendritic cells, which may be activated as antigen presenting cells (Getz, 2005), recent studies report the expression of TLRs in adaptive immune response cells in mammals (Bourke *et al.*, 2003; Reynolds *et al.*, 2010). Therefore, the immune system as a whole represents a very complex, interacting network.

### *Toll-like receptors*

One of the earliest indicators that the host has been infected with a pathogen is the activation of signaling pathways by TLRs (Kumagai and Akira, 2010). TLRs can be classified based on the types of ligands they recognize. Lipid-based pathogen-derived structures are recognized by TLR2 (with TLR1 or TLR6) and TLR4 (as a homodimer): the extensively studied lipid-based recognition molecules are bacterial or mycobacterial lipopeptides, or glycerophosphatidylinositol anchors from parasites, both of which are recognized by TLR2, and the bacterial lipopolysaccharide (LPS) which is recognized by TLR4. Viral or bacterial nucleic acids are recognized by TLR3, TLR7, TLR8 and TLR9; the most extensively characterized are the recognition of double stranded RNA (dsRNA) by TLR3 and recognition of CpG motifs in DNA by TLR9. Additionally TLR5 and mouse-specific TLR11 recognize proteins from pathogens: flagellin in the case of TLR5 and profilin in the case of TLR11 are the examples of these proteins (Akira *et al.*, 2003).

The cellular events that are activated through TLRs have been a subject of many research studies. Many biochemical details of these have been discovered including novel adaptor molecules, protein kinases and transcription factors. Although different TLRs use shared pathways, there are differences in patterns of inflammatory responses to various TLR agonists (Jones *et al.*, 2001). In a broad sense, there are two predominant intracellular TLR pathways. The MyD88-dependent (D) pathway uses the adaptor molecule MyD88 and leads to activation of NF- $\kappa$ B and production of cytokines; the MyD88-independent (I) pathway signals via the Toll-IL-1R domain-containing adaptor inducing IRF3 leading to IFN- $\beta$  production. The I pathway can also activate NF- $\kappa$ B but in a delayed manner. With the

exception of TLR3 and TLR4, all TLRs in mammals signal through the D pathway (Bagchi *et al.*, 2007). TLR4 is unique in that it activates both the D and I pathways in mammals (Toshchakov *et al.*, 2002; Yamamoto *et al.*, 2003b).

TLR signaling requires multiple components to be able to carry the signal from membrane to the nucleus to activate relevant transcription factors. Each TLR has a TIR (Toll/IL-1 receptor/resistance motif) domain. A total of five adapter proteins is currently known to convey signals from TLRs to cytosol. These adapters are MyD88, MAL (or Tirap), TRIF (or Ticam), MyD88-4 (sometimes called TIRP or TRAM) and MyD88-5 (Beutler, 2004).

### MyD88

MyD88 was the first identified adaptor molecule and like TLRs, it has a Toll-IL-1 receptor (TIR) domain; signaling is likely to be initiated by the recruitment of MyD88 to TLRs via TIR-TIR interactions (O'Neill, 2005). MyD88 knockout mice were not responsive to the TLR4 ligand LPS as evidenced by the inability of macrophages to produce inflammatory mediators, failure of B cells to proliferate and absence of endotoxic shock (Kawai *et al.*, 1999). These mice were also not able to respond to the TLR2 ligand peptidoglycan (Takeuchi *et al.*, 2000). Moreover MyD88 knockout mice showed no response to TLR9 agonist CpG or the TLR7 agonist imidazoquinoline (Hemmi *et al.*, 2002; Schnare *et al.*, 2000).

### IRAK

IRAK is a serine/threonine kinase associated with the IL-1 receptor, (Cao *et al.*, 1996). There are four known IRAK molecules: IRAK1, IRAK-2, IRAK-M, and IRAK-4. IRAK proteins carry an N-terminal death domain, which is required for interaction with MyD88, and a central kinase domain. IRAK1 knockout mice showed a partially impaired LPS response, whereas IRAK-4 knockout mice showed almost complete impairment in the response to pathogenic components that can stimulate TLR2, TLR3, TLR4, or TLR9 (Suzuki *et al.*, 2002). Interestingly, IRAK-M defective mice showed increased expression of inflammatory cytokines after stimulation with the TLR ligands and over-expression of inflammatory response genes to bacterial infection, suggesting that IRAK-M has negative or regulatory effects on the TLR signaling pathway (Kobayashi *et al.*, 2002a).

### TRAF

TRAF family members are considered as the downstream molecules of the TLR signaling pathway. TRAF proteins carry C-terminal TRAF domains (TRAF-N and TRAF-C), which interact with TRAF proteins and other signaling molecules, N-terminal RING finger, and zinc finger domains (Arch *et al.*, 1998). After stimulation with TLRs, TRAF6 is recruited to the receptor complex, and activated by IRAK1, which binds to the TRAF domain of TRAF6. After that, the IRAK1/TRAF6 complex released from the receptor and associates with TGF-beta-activated kinase 1 (TAK1) and TAK1-binding proteins, TAB1 and TAB2, at the membrane. IRAK1 stays in the membrane and is degraded while the TRAF6, TAK1, TAB1, and TAB2 complex goes into the cytoplasm, where it makes a large complex with other proteins including the E2 ligases Ubc13 and Uev1A (Deng *et al.*, 2000), and activates NF- $\kappa$ B. In addition to these downstream molecules, there are other molecules involved in the

TLR signaling. For example, Tollip has been shown to form a complex with IRAK1. Tollip was previously reported as part of IL-1 signaling (Martin and Wesche, 2002). IL-1 stimulation causes the formation of a Tollip-IRAK1 complex which is then recruited to the IL-1 receptor complex. This event leads to phosphorylation of IRAK1 and subsequent dissociation of IRAK1 from Tollip, which activates TRAF6. Although Tollip has been shown to negatively regulate the TLR-mediated signaling (Zhang and Ghosh, 2002), it remains unclear how Tollip is physiologically involved in TLR signaling.

As explained above, MyD88 knockout mice did not show any production of inflammatory cytokines after treatment with any of the TLR ligands. However, in the case of TLR4 stimulation, delayed LPS-induced activation of NF- $\kappa$ B was observed, even in MyD88 deficient cells. Although these cells did not express any inflammatory cytokines in response to LPS (Kawai *et al.*, 1999), expression of IFN-inducible genes was detected (Kawai *et al.*, 2001). These studies therefore demonstrated that there is a MyD88-independent pathway in addition to a MyD88-dependent pathway in TLR signaling. Analysis of the MyD88-independent pathway identified two TIR domain-containing adaptors: TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal) and TIR domain-containing adaptor inducing IFN- $\beta$  (TRIF)/TIR domain-containing adaptor molecule (TICAM-1) (Hornig *et al.*, 2001; Yamamoto *et al.*, 2002b).

#### TIRAP/Mal

TIRAP/Mal adaptor molecules' TIR domain is in the C-terminus. Preliminary *in vitro* studies reported that TIRAP/Mal interacts with TLR4, and is involved in the TLR4-mediated MyD88-independent signaling pathway. However, even in mice with defective MyD88 and

TIRAP/Mal, the TLR4 ligand-induced expression of IFN-inducible genes was not impaired. Therefore, TIRAP/Mal is critically involved in the MyD88-independent pathway, not in the MyD88-dependent pathway through TLR4 (Yamamoto *et al.*, 2002a). However, TIRAP/Mal knockout mice showed normal responses to the TLR3, TLR5, TLR7, and TLR9 ligands, but were defective in TLR2 ligand-induced inflammatory cytokine production. Therefore, these studies showed that TIRAP/Mal is required for the MyD88-dependent signaling pathway via TLR2 and TLR4, but not for MyD88-independent signaling (Takeda and Akira, 2004).

#### TRIF/TICAM-1

TRIF/TICAM-1 was identified from a database search and as a TLR3-associated molecule by two-hybrid screening (Oshiumi *et al.*, 2003). Unlike MyD88 and TIRAP/Mal, TRIF is a large protein consisting of 712 amino acids in humans. Overexpression of TRIF, but not MyD88 or TIRAP, induced activation of the IFN- $\beta$  promoter. RNAi-mediated knockdown of TRIF caused impairment in the TLR3 ligand-induced IFN- $\beta$  expression. Hence, these *in vitro* studies showed that TRIF is involved in the TLR3-mediated MyD88-independent pathway. In TRIF-impaired mice, TLR3-mediated expression of IFN- $\beta$  and IFN-inducible genes decreased (Yamamoto *et al.*, 2003a)

In addition to MyD88, TIRAP, and TRIF, a fourth TIR domain-containing adaptor, TIRP, was reported to be involved in the IL-1 receptor-mediated signaling pathway. However, it is not clear whether TIRP mediates the TLR signaling pathway (Bin *et al.*, 2003). Human TIRP protein comprises 235 amino acids, and the TIR domain is located in the central part of the protein. SARM is an additional protein that contains about 700 amino acids, and the TIR domain is in the C-terminal portion. SARM was identified as the fifth

adaptor protein specifically inhibiting the TRIF-mediated signaling cascade (Carty *et al.*, 2006; Piao *et al.*, 2009).

The discovery of TLRs in mammals expedited the process of understanding the molecular mechanisms of innate immunity. Each TLR recognizes its cognate microbial components and activates signaling pathways. The TLR signaling pathways also have their own cascades for demonstrating their distinct biological responses, which are characterized by several TIR domain-containing adaptors (Kawai and Akira, 2010). Exploitation of the physiological effects of these adaptors will provide more information as to how TLRs induce their specific innate immune responses.

#### NOD (Nucleotide binding and Oligomerization Domain)-like Receptors

Another pathogen recognition mechanism which is part of innate immunity, consists of Nod proteins. Nod1 and Nod2 are two cytosolic mammalian proteins and demonstrated to be intracellular peptidoglycan receptors (Girardin *et al.*, 2003a; Girardin *et al.*, 2003b; Totemeyer *et al.*, 2006). Nod proteins harbor a CARD domain at their amino termini, a single CARD domain in Nod1 and two tandem CARD domains present in Nod2, followed by a nucleotide-binding domain and a series of tandem leucine rich repeats (LRR). After peptidoglycans are recognized, Nod proteins interact with another CARD domain-containing protein Rip2 (or RICK) which has recently been reported to be necessary for the TLR-mediated activation of NF- $\kappa$ B (Kobayashi *et al.*, 2002b). This suggests a convergence of the signal pathways that are initiated by TLRs and Nod1. Furthermore, cytokine production in Rip2-deficient cells decreased after stimulation of TLRs with LPS, peptidoglycan or double-stranded RNA, but not with bacterial DNA, indicating that Rip2 is downstream of TLR2,

TLR3 and TLR4 but not TLR9. Rip2-deficient cells also showed impaired response in signaling through Nod proteins (Kobayashi *et al.*, 2002b). One important difference between the peptidoglycan recognition of Nod1 and Nod2 is that Nod1 recognizes peptidoglycan from Gram-negative bacteria, while Nod2 can recognize peptidoglycan from both Gram-negative and Gram-positive bacteria, difference being the biochemical structure of this component (Chamaillard *et al.*, 2003). Recent studies report that NLR genes are involved in inflammatory disorders such as 1) Crohn's disease and Blau syndrome (*Nod2*), 2) asthma and atopic disorders and inflammatory bowel disease (*Nod1*), 3) Muckle-Wells syndrome, familial cold autoinflammatory syndrome chronic infantile neurologic cutaneous and articular syndrome, inflammatory bowel disease (*Nlrp3*), and 4) vitiligo (*Nlrp1*) (Geddes *et al.*, 2009), however the mechanisms of action have not been studied in detail.

### *Adaptive Immunity*

Immune system is the result of interplay between two systems: adaptive and innate. The immune system of higher vertebrates demonstrates delicate specificity and strong immunological memory. These are the essential characteristics of adaptive immunity which is remarkable for its ability to improve and change over time. The adaptive immune response is antigen-specific and may take days or longer to develop. The system (for B cells) is very adaptable because of somatic hypermutation (accelerated somatic mutations) and V (D) J recombination (irreversible genetic recombination of antigen receptor gene segments). These mechanisms generate a wide range of different antigen receptors that are expressed on each individual B lymphocyte. The gene rearrangement leads to an irreversible change in the genome of each cell, which then passes onto the progeny who have the receptor specificity,



including the memory B cells and memory T cells that are the keys to long-lived specific immunity. Immunological memory is the primary feature of adaptive immunity. Therefore, the organism remembers the original pathogen and mounts humoral and cellular responses to control the reinfection and fight the disease (Cui and Kaech, 2010). Immunological memory consists of long-lived plasma cells that secrete high affinity antibodies, and memory B and T cells (Kaech et al., 2002).

### **Comparison of innate and adaptive immunity**

There are many distinguishing characteristics of the innate and adaptive immune systems, but the most important difference is genetic (Table 1). The recognition proteins of the innate immune system are germ-line encoded, having evolved in invertebrates to fight infections (Rasmussen *et al.*, 2009). These recognition proteins have been selected for their ability to recognize pathogens and to initiate a host response. Thus, innate immunity provides evolutionary evidence for the biologically relevant structures which interact with specific infectious organisms. However, innate immunity has the disadvantage of being able to recognize only a few highly conserved microbial structures which evolve relatively slowly compared to microorganisms. This problem is overcome by the receptors of the adaptive immune system, such as B and T cells, that are not encoded in the germline. Rather, they are products of somatic rearrangements (V, D, and J segments of T cell receptors and antibody genes). This remarkable arrangement happened suddenly with the evolution of vertebrates 400 million years ago and generates a large repertoire of antigen binding structures (Fearon, 1999). Somatic selection can be thought as a form of education (the phase when T cells are trained to recognize the desired molecules) about which antigen receptors are biologically

relevant. This selection takes place in two stages, the first is positive and the second is negative selection of developing lymphocytes. This way, highly self-reactive receptors are eliminated, which is called negative selection, and the ones that interact with antigens lead to an effective immune response, which is called positive selection (Table 1.).

Genes involved in the regulation of immune responses are generally responsible for the control of invading pathogens and, therefore, are subject to the problem of the rapid evolution of escape mechanisms of pathogens. For this reason, pathogens have evolved strategies to evade host immune response for their survival and transmission (Coombes and Robey, 2010). This selection pressure gives rise to great sequence diversity in the immune response genes in different species. There are fundamental differences between the immune systems of birds and mammals including the arrangement of the MHC (Kaufman *et al.*, 1999), different mechanisms of somatic recombination in the generation of antibody diversity (Reynaud *et al.*, 1989) and the absence of lymph nodes in birds (Kaiser *et al.*, 2005). In some aspects, immune system of chicken might be considered a minimal version of that in mammals. Chickens have a reduced repertoire of granulocyte populations, neutrophils, eosinophils and basophils, which are effectively replaced by the heterophils (Kaiser *et al.*, 2005). Chickens also lack lymph nodes, which are primary site of antigen presentation in mammals. These differences lead to the expectation of a reduced repertoire of cytokines and chemokines in chickens. However, chickens overcome this with specialized lymphoid tissues and patterns of development that do not exist in mammals, such as the bursa of Fabricius where the B cells are produced, by means of gene conversion, for immunoglobulin diversity, and the much larger complement of  $\gamma\delta$  T cells (Arstila and Lassila, 1993). Regulation of

these cells might require a special set of cytokines whose function is not part of the mammalian immune system.

Cytokines are glycopeptide molecules that are involved in the coordination of the immune response and expressed and secreted by many different types of cells. They show their effects on both homeopoietic cells and immune system cells that are involved in host defense and homeostasis. The sets of cytokines includes interleukins (IL), interferons (IFNs), colony-stimulating factors (CSFs), transforming growth factors (TGFs), tumor necrosis factors (TNFs) and the small peptide chemokines, all of which exist in mammals. New members of the cytokine families have been discovered since the genome sequences of human, mouse, and the chicken became available. Nomenclature for what changes as the functional and structural characteristics of cytokines are elucidated. For example IL28A, IL28B and IL29 recently were shown to form a new family of type I IFNs, and have been named IFN-delta (Kaiser *et al.*, 2005).

There are important differences between chicken and mammalian IL families. For example, the chicken IL10 family is smaller than the human IL10 family. In humans, there are six IL10 family members (IL10, IL19, IL20, IL24, IL22, and IL26) (Fickenscher *et al.*, 2002) whereas the chicken IL10 family has three members (IL10, IL19, and IL22) (Kaiser *et al.*, 2005). Additionally, the human genome sequence has provided evidence for the existence the IL17 family, with 6 members (IL17A-F) (Moseley *et al.*, 2003), but there is no evidence for chicken orthologues of IL17C or IL17E.

Chickens have approximately 10 type I IFNs. Three of these were sequenced and don't have any introns (IFNA 1-3). The predicted proteins have 24% homology in amino

acid sequence with mammalian IFNA and only 3% with IFN $\gamma$  (Sick *et al.*, 1996). Annotation of the chicken type I IFNs in the genome is not very definitive because IFNA and IFN $\gamma$  genes are on the Z (sex) chromosome of which the chromosome assembly is currently poor in chickens (Nanda *et al.*, 1998). Despite the low sequence identities shared with mammalian counterparts, chicken IFN $\gamma$  shows a high similarity in the regions of the core structure. The function of IFN $\gamma$  in chickens and fish are similar and comparable with mammals, suggesting that conservation of the biological function of IFN $\gamma$  gene, during evolution, represents the dynamism of a gene subjected to strong selective forces (Savan *et al.*, 2009).

### **Chickens: macrophages, Toll-like receptors and *Salmonella***

Monocytes-macrophages belong to the mononuclear phagocytic system and are considered the first line of host defense. These scavenger cell types derive from bone marrow stem cells by differentiating into monoblasts, promonocytes and monocytes. While monocytes form a major phagocytic cellular component in blood, tissue macrophages are widely distributed and present in almost every organ (Geissmann *et al.*, 2010). The chicken abdominal cavity is the most common source to isolate macrophage cell populations. Unlike mice, chickens do not have resident macrophages in their abdominal cavity (Sabet *et al.*, 1977). However, macrophages can be activated to migrate to this site if an appropriate stimulant is employed intraabdominally. An effective inflammatory stimulant in chickens is Sephadex<sup>®</sup>. A single injection of 3% Sephadex<sup>®</sup> can attract numerous inflammatory macrophages to the abdominal cavity. The cells collected at 40 hours post Sephadex<sup>®</sup> stimulation consist of > 97% macrophages (Qureshi *et al.*, 1986). During an inflammatory response, macrophages undergo maturation over time. Sephadex-recruited macrophages

collected at 6 hours post Sephadex<sup>®</sup> stimulation are not able to phagocyte unopsonized targets efficiently. On the other hand, if the harvesting time is 40 hours after Sephadex<sup>®</sup> stimulation, the phagocytic potential increases about 40 fold (Chu and Dietert, 1988). This phenomenon might be explained by the increased expression of Fc receptor on the cellular membrane of the macrophages (Qureshi *et al.*, 2000).

Transformed chicken macrophage cell lines are another resource to study macrophage function. The most commonly used avian macrophage cell lines are MQ-NCSU (Qureshi *et al.*, 1990) and HD11 cells (Beug *et al.*, 1979) which are transformed with an avian myelocytomatosis virus MC29. Several avian RNA viruses have been reported to transform chicken macrophages. Functionally mature macrophages are susceptible to transformation by avian myeloblastosis virus (AMV). This transformation is not dependent on the virus replication. The population of target cells for transformation is quite low because of the decreased proliferative activity of macrophages in cultures (Qureshi *et al.*, 2000). The AMV and MC29 viruses have been demonstrated to suppress different markers and certain functions of these transformed cells. In contrast, cells infected with the Rous sarcoma virus (RSV) and myeloblastosis-associated virus (MAV-2) did not show altered cell phenotype or growth rate or expression of differentiated functions of macrophages (Qureshi *et al.*, 2000). Oncogenes associated with various viruses including MC29, OK10, CMII and MH2, four acute leukemia virus strains, induce macrophage transformation. For example, the MH2 efficiently induces monocytic leukemia and liver tumors; its two oncogenes, *v-myc* and *v-mil*, have to incorporate for stable transformation of homeopoietic cells into a macrophage like phenotype. The MH2 retrovirus oncogene *c-myc* stimulates cell division, while *v-mil* induces

the chicken myelomonocytic growth factor (cMGF) (Beug *et al.*, 1981). Therefore, macrophages transformed with MH2 virus will proliferate without growth factor addition to the medium because of an intrinsic growth system. The bone marrow derived HD11 cells were transformed with MC29 virus based on the same principle and were chosen for use in the current study because of their uniformity and inducibility in cultures and large body of literature documenting their use in immunology studies (Brownlie *et al.*, 2009; Karpala *et al.*, 2008).

#### *Avian Toll-like Receptors*

Toll-like receptors were first described in mammals after toll protein was described in *Drosophila* in 1985 by Christiane Nüsslein-Volhard. Mice have 12 (1-9 and 11-13) and humans have 10 (1-10) TLRs. Although aves and mammals diverged 300 million years ago, the avian TLRs family contains both orthologous and distinct TLR genes. Ten avian TLRs have been identified to date, and they are clear orthologs of mammalian TLR3, TLR4, TLR5, and TLR7, which are encoded in conserved genomic regions (Boyd *et al.*, 2007).

Phylogenetic studies show that six (TLR2, 3, 4, 5, 6, 7) of these genes are also present in mammals, one is shared by fish (TLR21) and three are unique to birds (TLR15, TLR1likeA and TLR1like B) (Temperley *et al.*, 2008). TLR8 gene is fragmented by a Chicken Repeat-1 (CR1) retrovirus-like element. Because there is no protein encoded by this gene, it is a pseudogene. There is no evidence for a chicken TLR9; it is not located in the genomic region conserved with mammals. However chicken cells are able to respond to CpG-oligonucleotides, the ligand of mammalian TLR9 (Mackinnon *et al.*, 2009). Orthologs of murine TLR11, 12, 13 have not been identified in chicken genome (Table 2). TLR15 is not

related by structure to any known vertebrate TLR. The degree of amino acid sequence similarity between chicken TLRs and orthologous mammals TLRs is high, compared to other immune-related genes (Boyd *et al.*, 2007). Functional analyses, as well as phylogenetic and *in silico* analyses, showed that chicken TLR3 is responsive to poly I:C treatment (Karpala *et al.*, 2008), chicken TLR4 to LPS (Keestra and van Putten, 2008), chicken TLR5 to flagella (Nerren *et al.*, 2010), TLR7 to agonist R848 and loxoribine (Philbin *et al.*, 2005) and that chicken cells are able to respond to CpG-ODN in spite of the absence of TL9 (Jenkins *et al.*, 2009; Mackinnon *et al.*, 2009).

Nod-like receptors (NLRs) have recently been discovered in chickens (Ciraci *et al.*, 2010; Li *et al.*, 2010) as well as in mammals (Totenmeyer *et al.*, 2006). The exact mode of NLR activation in the host cytosol remains poorly defined, because evidence of direct interaction between NLRs and pathogen-associated molecular patterns remains to be elucidated. However, recent concurrent observations suggest that activation of some NLRs occurs next to host membranes or as a result of membrane damage (Philpott and Girardin, 2010). The mammalian NLRC5 gene has recently drawn considerable attention. Its functions as a transcriptional regulator of MHC class I, as a suppressor of NF- $\kappa$ B and type I IFN signaling and involvement in antiviral responses have recently been reported (Benko *et al.*, 2010; Meissner *et al.*, 2010; Neerincx *et al.*, 2010). The current dissertation represents the first report of the chicken CARD domain containing NLR family member, NLRC5, expression in response to *Salmonella typhimurium* derived endotoxin.

*Salmonella*

Salmonellosis is a zoonotic disease caused by the Gram negative facultative enteric bacterium *Salmonella*. Different serotypes of *Salmonella* can infect wide range of domestic animals including poultry, sheep, cattle, sheep and pigs and cause varying symptoms ranging from gastro-enteritidis to death. *S. typhimurium* and *S. enteritidis* can also infect humans. Poultry represent a major source of *Salmonella enterica* food poisoning in human, mostly associated with these serotypes (Beal *et al.*, 2004). Some serotypes are host-specific, such as *S. Gallinarium* and *S. Pullorum*, which infect single species and sometimes lead to death. These serotypes can cause disease outbreaks resulting in severe economic losses (Calenge *et al.*, 2010). Many strategies aimed at reducing infection of chickens by these serovars have been developed. As food safety became an important concern, eradication, sanitation, reducing environmental stressors and use of antibiotics and vaccines were employed to obviate the losses caused by disease outbreaks. The poultry industry is seriously affected by infectious disease and contamination of food products as a result of the transfer of microorganisms through eggs and meat. However, there is no commercially available chicken line bred for polyvalent resistance to multiple infectious agents (Swaggerty *et al.*, 2009). Public outcry against the use of broad spectrum antibiotics for disease control or for growth promotion in chickens will limit the traditional management of infectious poultry diseases using antibiotics as a routine husbandry practice.

The desired outcome of a host-pathogen interaction is clearly the destruction of the pathogen. Macrophage responses to different bacteria at the level of transcription are not well understood. Considering the cellular components shared between bacteria and the signaling pathways shared by TLRs that are able to recognize these bacterial components,



macrophages may respond to most bacteria in a standard manner. However, the diversity of bacteria and the discrepancies in their pathogenesis might lead to specific responses.

*Salmonella enteritidis* is similar to *Salmonella typhimurium* regarding the known virulence mechanisms in mammalian cell invasion, survival and growth in the host cell. Both *S. enteritidis* and *S. typhimurium* have the highly conserved pathogenicity island-encoded type III secretion mechanisms and virulence effector proteins. Both bacteria produce a galactose-rhamnose-mannose repeat unit of the lipopolysaccharide (LPS)-O-chain backbone decorated with a dideoxyhexose that determines serotype (Marcus *et al.*, 2000; Suarez and Russmann, 1998). Lipopolysaccharide extracted from *Salmonella enteritidis* differs between strains in the level of glycosylation of the O-chain region, meaning that glycosylation ranges from a lack of glycosylation in stored isolates to high levels as recovered from variants freshly cultured from the spleen of naturally infected mice. On the contrary, glycosylation of LPS from *S. typhimurium* does not change, even when the strains have been stored for years (Parker *et al.*, 2001).

### **RNA interference (RNAi)**

One of the most important advances in biology in decades has been the discovery of RNA molecules that can regulate the gene expression (Fire *et al.*, 1998). RNAs were initially thought to possess two broad functions. First, single stranded messenger RNAs are essential intermediate molecules in gene expression, conveying information from DNA to protein. Second, ribosomal and transfer RNAs have structural, catalytic and information deciphering roles during translation. In 1998 Andrew Fire and Craig Mello reported their discovery of RNA interference, which is the silencing of gene expression by injection of double stranded

(ds) RNAs into *Caenorhabditis elegans* (Fire *et al.*, 1998). Since then, this RNA picture has become more complete and complicated (Novina and Sharp, 2004). Double stranded RNA was found to knockdown gene expression by binding to their complementary sequences on mRNA for degradation. The studies reported in last decade revealed that the long dsRNAs were diced up into short RNAs with a specific structure: two 21-nucleotide strands of RNA in a duplex, with 19 nucleotides of dsRNA and two unpaired overhanging nucleotides at the ends and synthetically synthesized siRNAs were also shown to knockdown genes (Elbashir *et al.*, 2001). The long dsRNA might be produced from an introduced transgene including repetitive genes or a viral invasion. Therefore, RNAi might be considered as the “immune system of the genome” by surveying (monitoring) and protecting against dsRNA invasion by viruses, transposons and aberrant nonsense mRNAs (Tijsterman *et al.*, 2002).

The enzyme that cleaves the dsRNA into siRNA is called Dicer (Bernstein *et al.*, 2001). Short interfering RNAs (siRNAs) silence genes by promoting cleavage of mRNA with exactly complementary sequences or recruiting inhibitory proteins to, or directing the modification of, DNAs with exactly complementary sequences. When the long dsRNA is cut into siRNAs, an RNA-induced silencing complex (RISC) then determines the sense strand which is degraded upon recognition by RISC.

RISC consists of DICER, TRBP (the human immunodeficiency virus transactivating response RNA-binding protein) and argonaute proteins (Chendrimada *et al.*, 2005; Das *et al.*, 2006). Argonaute proteins, reported from RISC complexes of diverse organisms, are the key components of RISC. The Argonaute protein family is highly diverse, and the members harbor two domains: a PAZ domain, which is involved in miRNA/siRNA binding, and a

PIWI domain, which is related to RNaseH endonucleases and functions in slicer activity (Tahbaz *et al.*, 2004).

RNAi is an endogenous cellular process by which messenger RNAs are targeted for degradation by dsRNA of identical sequence, leading to gene silencing (Chapman and Carrington, 2007). RNAi has become a powerful tool to silence gene expression at the post-transcriptional level for studying gene function. This sequence-specific gene-silencing technique which is mediated by double-stranded RNA, has opened new avenues for genetic screens in various model organisms such as nematode worms, birds and mammalian cells (Cheeseman *et al.*, 2008; Fire *et al.*, 1998; McManus and Sharp, 2002).

Conventionally, functional studies are classified into forward and reverse screens. A typical forward genetic research study starts with the generation of random mutants to produce a specific phenotype, and the next step would be the identification of genetic change that affected the phenotype. In mammalian cells, the time required to finalize the study is usually estimated between 6 months to 1 year. On the other hand, the reverse genetics involves the selective downregulation of a gene function. Although knockout techniques generates the complete inhibition of a protein by disruption of two alleles of the genome (homologous recombination), the time necessary to complete the process is also 6 months to 1 year. However, RNAi technology can achieve up to 80-90% suppression of the gene expression in shorter periods of time, such as weeks. Therefore, siRNAs are faster, simpler and more cost effective alternatives for studying gene functions in a sequence-specific manner (Silva *et al.*, 2004).

Although many studies focusing on gene function have reported the use of RNAi technology in mammalian systems, few studies have been done in the chicken. One of the early RNAi studies in chicken cells was reported by Cheeseman *et al.*, (2008) and showed the applicability of this useful tool in chicken cells. Another study in chickens investigated the roles of TLR3 and its effects on the production of IFNs utilizing siRNAs (Karpala *et al.*, 2008). Additionally, the function of chicken TLR21, involvement of NKB1 in response to *Salmonella* in chicken macrophage HD11 cells by RNAi were demonstrated (Brownlie *et al.*, 2009; Han *et al.*, 2009). One of the most recent studies in chicken also utilized RNAi technology for developing transgenic chickens resistant to avian influenza virus (Zhang *et al.*, 2010).

One factor for successfully using RNAi technology is the effective delivery of siRNA to the target cells or tissues. Naked siRNAs do not freely diffuse across the cell membrane due to their large molecular weight and polyanionic nature, so a delivery system is required to facilitate siRNA entrance to its intracellular site of action. Different approaches have been noted for both *in vivo* and *in vitro* delivery of siRNAs. Broadly, these delivery strategies can be divided into nonviral and viral delivery methods. Nonviral delivery methods involve the use of unmodified synthetically synthesized siRNAs/shRNAs, *in vitro* transcribed siRNAs, or plasmid vector expressed siRNAs/shRNAs/dsRNAs. Delivery can be mediated by liposomes, lipids, protein-antibody conjugates and peptides. Short hairpin (sh) RNAs act as substrates for dicer and gives rise to siRNAs. The loop structure in shRNAs might contribute in enhancing the RNAi (Brummelkamp *et al.*, 2002). The viral delivery method can be more advantageous for cells that are difficult to be transfected by other methods and can even be

used in nondividing cells. The viral vectors transduce cells naturally and exert very high transduction efficiency compared to nonviral methods (Singh and Hajeri, 2009).

Regardless of the delivery methodologies of synthetic siRNAs into cells, they can have off-target effects (Cullen, 2006). Both siRNAs and shRNAs might contain stretches of dsRNAs, in which case they can activate nonspecific innate immune responses, such as the interferon response. Transfected or expressed siRNAs can also show other nonspecific effects; for example, artificial siRNAs or shRNAs could saturate the RNAi machinery of cell and thereby inhibit the function of endogenous miRNA. In addition, transfected siRNAs having certain sequence motifs can activate the innate immune system by engaging Toll-like receptors (Hornung *et al.*, 2005). Therefore, it is crucial to avoid non-specific or specific off-target effects during experiments. To minimize the probability of specific off-target and non-specific effects, siRNAs should be verified to be fully homologous to the desired mRNA target in expressed sequence tag (EST) database for the relevant organism. It has been noted that siRNA stretches corresponding to various intron and promoter sequences did not produce detectable interference (Fire *et al.*, 1998), therefore this also should be taken into consideration while designing siRNAs directed at the gene of interest.

Research in genetic control of disease resistance involves not only structural variation of associated genes and their products but also variation in gene expression (Lamont, 1994). The host immune response to pathogens in the earliest phases of the infections is essential for the determination of disease resistance and susceptibility. Functional genomics studies could provide information on host defense mechanisms with regard to genes differentially expressed between animals or cells that have (versus have not) been exposed to pathogens or

pathogen components. Elucidation of the mechanisms acting on the transcriptional regulation of host response, such as receptor/ligand engagement, adaptor molecules involved in the activated signaling pathways, receptor/ligand redundancy, the differential expression of immune response genes, and kinetic genome-wide gene expression pattern in activated macrophages will contribute to a better understanding of chicken immunity in the context of animal health and food safety. Application of this understanding could include development of effective immunomodulatory treatments or genetic selection for more innate resistant genotypes.

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**TABLES:**

Table 1. Comparison of general characteristics of innate and adaptive immunity (Borghesi and Milcarek, 2007; Janeway and Medzhitov, 2002)

<u>Characteristics</u>	<u>Innate immunity</u>	<u>Adaptive immunity</u>
Receptors	Fixed in genome Rearrangement is not necessary	Encoded in gene segments, rearrangement is necessary
Distribution	Non-clonal All cells of a class identical	Clonal All cells of a class distinct
Self-nonself discrimination	Perfect: selected over evolutionary time	Imperfect: selected in individual somatic cells
Memory	No	Yes
Specificity	Degenerate	Specific
Response Kinetics	Rapid	Delayed

Table 2. Avian TLRs and mammalian counterparts, chromosome location, ligand and gene accession numbers [modified from Akira and Takeda, (2004); Temperley *et al.*, (2008)] (NI: not indentified)

<u>Avian TLR</u>	<u>Mammalian TLR</u>	<u>Avian Chr #</u>	<u>Ligand</u>	<u>Genbank Accession no. (<i>Gallus gallus</i>):</u>
TLR1LA	TLR1	4	Unknown in chicken; lipoprotein in mammals	AB109401
TLR1LB		4	unknown	DO518918
TLR2A	TLR2	4	Lipopeptide peptidoglycan	NM_204278
TLR2B		4	Lipopeptide peptidoglycan	AB046533
TLR3	TLR3	4	dsRNA	NM_001011691
TLR4	TLR4	17	LPS	AY064697
TLR5	TLR5	3	Flagellin	AJ626848
TLR6	TLR6		Diacyl lipoproteins	
TLR7	TLR7	1	Imiquimid	NM_001011688
Pseudogene	TLR8		ssRNA in mammals	
NI	TLR9		CpG-ODN	
NI	TLR10		unknown	
NI	TLR11		Profilin like protein	
	Not in human			
NI	TLR13		Similar to TLR21	
	Not in human			
TLR15	N/A	3	CpG-ODN Heat-killed <i>Salmonella</i> Typhimurium	XM_419294
TLR16	NI		Covers for TLR1 and TLR6	
TLR21	NI	11	CpG-ODN	NM_001030558



## Chapter 2: Avian-specific TLRs and downstream effector responses to CpG-induction in chicken macrophages

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### Abstract

Chickens possess toll-like receptor (TLR15), a pattern recognition receptor (PRR) absent in mammals. We characterized the regulation and mechanism of CpG responsiveness via TLRs in chicken macrophage HD11 cells. TLR15 was significantly upregulated after induction with B- and C- type CpG oligonucleotides (ODN), tripalmitoylated lipopeptide (PAM3CSK4), *Escherichia coli*- and *Salmonella enteritidis*-derived lipopolysaccharide (LPS). In response to CpG-ODN inhibitor, TLR15 and IL1  $\beta$  were downregulated, but TLR21 was upregulated. IL1B was upregulated with CpG-ODN and downregulated after inhibitor treatment. The results suggest that responsiveness to different types of CpG-ODN in chicken macrophages requires multiple receptors, each with unique variation in expression. We utilized RNA interference (RNAi) technology to examine myeloid differentiation primary response gene (MyD88) dependency of TLR15 and TLR21. HD11 macrophages transfected with multiple MyD88-target siRNAs exhibited 70% decrease in MyD88 mRNA expression. IL1B was upregulated with CpG induction in cells with no reduction of MyD88 mRNA levels, but not in cells with 70% MyD88 reduction. Therefore, induction through

TLR15 in response to CpG-ODN operates via the MyD88-dependent pathway in chicken macrophages.

## **Introduction**

Innate immune response in chickens is mediated mainly by leukocytes such as heterophils and macrophages. Chicken macrophages have important regulatory functions in host immune response because they are dispersed all through the bird's body fluids and tissues and are capable of producing a wide range of inflammatory mediators and cytokines as do their mammalian counterparts (Klasing, 1998). Regulatory roles of chicken macrophages are vital for determination of the type and intensity of innate and specific immune responses. Although TLRs are known to be innate immune response receptors that recognize pathogen associated molecular patterns (PAMPs) (He et al., 2006), recent studies demonstrate the expression of TLRs in acquired immune response cells in mammals (Bourke *et al.*, 2003; Reynolds *et al.*, 2010). Therefore, engagement of TLRs with their cognate ligands leads to intricate and multifaceted immune responses.

Although mammals and birds diverged about 300 million years ago, there are evolutionarily conserved regions on the chromosomes of both classes (Burt *et al.*, 1995) including genes encoding Toll-like receptor (TLR) family members (Philbin et al., 2005). Structural and phylogenetic analysis of TLRs demonstrates that chickens have a total of ten TLRs, six of which have orthologs in mammals and fish. TLR21 is shared only with fish; and TLR1likeA, TLR1likeB and TLR15 are unique to birds. The TLR7, 8 and 9 subfamily is

present in fish and mammals but is only represented by TLR7 in the chicken. TLR8 is a pseudogene and TLR9 has been proposed to have been deleted from the avian genome over evolutionary time (Temperley et al., 2008). The presence of TLR15 in only avian species might be a result of gene gain over evolutionary time to compensate the missing TLRs in chickens. TLR15, as a molecularly distinct receptor from all previously discovered TLRs, has the closest amino acid sequence identity (30%) to chicken TLR2 (Higgs et al., 2006; Roach et al., 2005). Additionally, sequence comparison analysis revealed that chicken TLR21 (previously reported as the functional homologue of human TLR9 (Brownlie et al., 2009)) is most similar to TLR21 of *Xenopus tropicalis* (61%) and *Takifugu rubripes* (57%) and, more distantly, to murine TLR13 (47%). Comparative analysis of the protein sequence of chTLR21 and mammalian TLR9 demonstrate substantial additional differences in protein architecture (Keestra et al., 2010).

Research over the past few decades suggests that nucleic acids also function as stimulators of a rapid immune response when they are released from pathogens. Their link to pathogens attracted more attention after the discovery of TLRs (Akira and Takeda, 2004; Medzhitov and Janeway, 2002). CpG-oligonucleotides (ODNs) are utilized for a variety of therapeutic purposes, such as immune adjuvants, for cancer therapy and as anti-allergens (Takeshita et al., 2004). Additionally, treatment of chickens with CpG-ODNs is alternative to antibiotics because of their roles in increasing resistance to disease-causing pathogens (Mackinnon *et al.*, 2009). CpG-ODNs have been widely used in avian immune response studies that quantified TLR mRNA levels in chicken B cells (DT40), macrophages (HD11),

and primary heterophil cell cultures. However, previous studies were carried out using CpG-ODNs containing the identical CpG motifs (Han *et al.*, 2009; He *et al.*, 2007; Mackinnon *et al.*, 2009; Nerren *et al.*, 2010). One exception to these reports was the study by (Xie *et al.*, 2003) which used a different CpG motif, containing B type CpG-ODN in HD11 cells; however, the TLR response in HD11 cells was not assessed in that study. Different types of CpG-ODN ODN have distinct and, in some cases, opposite effects on the same cells, a finding with important applications for therapeutic use of these agents (Gursel *et al.*, 2002).

Despite the evidence of TLR15 induction following *S. typhimurium* infection in chicken embryonic fibroblasts (Higgs *et al.*, 2006) and *S. enteritidis* in primary heterophils (Nerren *et al.*, 2010), a specific structural stimulant for TLR15 and the relevant adaptor molecule that activates the downstream effectors have not been characterized. The present study represents a unique report because of the inclusion of different classes of CpG-ODN with different CpG motifs and the demonstration of the relations among the receptors (TLR15, TLR21, and TLR2), adaptor molecule (MyD88), and cytokine (IL1  $\beta$ ) in response to CpG-ODNs in chicken macrophages.

TLRs selectively use adaptor molecules such as MyD88, TRIF, TIRAP and TRAM to operate overlapping but different signaling pathways that lead to production of proinflammatory cytokines and interferons. Among all adaptor molecules, MyD88 is the most extensively studied one which is recruited to the cell membrane and interacts with TLRs through the TIR domain in mammals (An *et al.*, 2010). The differential engagements of TLRs with the stimulators give rise to predictable cytokine products based on MyD88

dependency. The My88 dependent pathway triggers the expression of proinflammatory cytokines such as IL6, IL10, IL1B while the MyD88 independent pathways activates type I interferon such as IFNA and IFN $\beta$  (Bagchi *et al.*, 2007).

TLR4 is a unique receptor in the TLR family in that it uses both MyD88-dependent and MyD88-independent signaling to initiate cytokine production in mice (Toshchakov *et al.*, 2002; Yamamoto *et al.*, 2003). A total of five cytoplasmic adaptor proteins that carry signals from TLRs into cytoplasm have been reported in mammals. However, only three (MyD88, Tirap and Ticam-1) of these five adaptor proteins have sequence information available in chickens. The type of signaling pathway that is activated by TLRs is important for the nature of the innate immune response (Beutler, 2004). Therefore, we examined the effects of different types of CpG-ODNs on the rapid chicken immune response via specialized receptors through subsequent differential gene expression in TLR signaling using chicken macrophage HD11 cells.

## **Materials and methods**

### **Cell culture and stimulation of cells with TLR agonists**

The chicken HD11 macrophage cell line, (Beug *et al.*, 1979), was cultured in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated newborn calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 10 mM HEPES and  $5 \times 10^{-5}$  M 2-mercaptoethanol (pH 7.3) at 40 °C and 5% CO<sub>2</sub>. Cells were plated in 75 cm<sup>2</sup> tissue flasks (Cellstar, Greiner Bio-one) and cultures were split every 3 days. Cell viability was > 90% by trypan-blue exclusion (Sigma-

Aldrich Co.). TLR agonists were dissolved in endotoxin-free H<sub>2</sub>O. Cells were cultured at an initial density of  $2.5 \times 10^6$  cells/flask into 25 cm<sup>2</sup> tissue flasks and kept overnight in the incubator, then stimulated with 0.0 (endotoxin-free H<sub>2</sub>O), 0.1, 1.0, 10.0 µg/ml doses of each of the following: PAM3CSK4 synthetic bacterial protein, peptidoglycan, ODN-2395 Type C CpG-ODN oligonucleotide, CL075 Thiazoloquinoline compound, Polyinosinic:polycytidylic acid (Poly (I:C)) synthetic analog of dsRNA , peptidoglycan from *Staphylococcus aureus* (PGN-SA) (Invivogen), lipopolysaccharide (LPS) from *Salmonella enteritidis*, LPS-*E.coli* K-325 (Sigma-Aldrich); then cells were collected at 3 and 24 hours after stimulation.

#### **Stimulation of cells with Type A, B and C CpG-ODN oligonucleotides (ODN) and inhibitor of CpG-ODN**

Cells were cultured at an initial density of  $2.0 \times 10^6$  cells/well into 6 well plates and kept overnight in the incubator, then stimulated with 10.0 µg/ml ODN 1585 (Type A: 5'-GGGGTCAACGTTGAGGGGGG-3'), ODN 1668 (Type B: 5'-CCATGACGTTTCCTGATGCT-3' ) or ODN 2395 (Type C: 5'-TCGTCGTTTTTCGGCGC:GCGCCG-3') (Invivogen) for 3 hours. Inhibition of CpG-ODN ODN stimulation was optimized including 1:1, 1:5, 1:10 ratio of inhibitory ODN: stimulatory ODN (G-ODN: 5'-CTCCTATTGGGGGTTTCCTAT-3'). Cells were incubated for 3 hours then harvested for RNA isolation.

#### **Real-time RT-PCR**

Total RNA was isolated from samples (3 individual 25 cm<sup>2</sup> flasks or one well of 4-well plates per treatment, 3 replicates per each treatment) using RNAqueous<sup>®</sup> (Ambion, Austin, TX) according to manufacturer's instructions. All RNA samples were DNase treated with DNA-Free (Ambion, Austin, TX) according to manufacturer's instructions before QPCR.

The mRNA expression levels of TLR15 (Higgs *et al.*, 2006), TLR21 (Brownlie *et al.*, 2009), TLR2 primers (F 5'-CTGGGAAGTGGATTGTGGA-3', R 5'-AAGGCGAAAGTGCGAGAAA-3'), TLR4 primers (F 5'-GGATCTTTCAAGGTGCCACA-3'R, 5'-CAAGTGTC CGATGGGTAGGT-3'), IL-10 primers (F 5'-CATGCTGCTGGGCCTGAA-3', R 5'-CGTCTCCTTGATCTGCTTGATG-3'), IFNA primers (F 5'-GACAGCCAACGCCAAAGC-3', R 5'-GTCGCTGCTGTCCAAGCATT-3'), IL1B primers (F 5'-GCTCTACATGTCGTGTGTGATGAG-3', R 5'-TGTCGATGTCC CGCATGA-3') and 28S primers ( F 5'-GGCGAAGCCAGAGGAAACT-3', R 5'-GACG ACCGATTTGCACGTC-3') as a housekeeping gene were determined by quantitative real-time RT-PCR , using QuantiTect SYBR Green RT-PCR (Qiagen, Waltham, MA). Each RT-PCR reaction was run in triplicate and consisted of either 50 ng/μl total RNA, 12.5 ml QuantiTect SYBR Green master mix, 0.25 ml QuantiTect RT mix, forward and reverse primers, and RNase-free water for a final volume of 25 ml. The QPCR reactions were performed on an Opticon 2 (MJ Research Inc., Waltham, MA). An initial 50°C step for 30 min was followed by 95°C for 15 min and 40 cycles (94 °C for 15 s, 59°C for 30s, and 72°C for 30s, for denaturation, annealing, and extension, respectively) for all PCR amplifications. Gene slopes were

determined with 10-fold serial dilutions. A melting curve from 60 to 90 °C with a reading at every 1 °C was also performed for each individual RT-PCR plate. Normalized cycle threshold C (t) values were calculated as follows:

$40 - [C(t) \text{ sample mean} + (C(t) \text{ 28s median} - C(t) \text{ 28s mean})] * (\text{gene slope}/28\text{s slope})$  for all genes. Mean adjusted C (t) values of each triplicate of assays were used in statistical analysis. To calculate differences between treatments,  $\Delta C_t$  values (i.e., the difference in  $C_t$  value between non-stimulated and stimulated macrophages) were compared. Results were expressed as fold-change in expression of stimulated cells relative to non stimulated cells.

### **Statistical analysis**

Expression levels of mRNA for each gene were analyzed with the JMP software (SAS Institute, Cary, NC) ANOVA model. The main fixed effects were post-agonist exposure harvest time (3, 24 hours) and TLR-agonist dose (0.0, 0.1, 1.0, 10.0 µg/ml) and the interaction of time and dose. Significant differences were ranked using Tukey Kramer Honesty test. *MyD88 siRNA*

### **Construction and transfection**

Three siRNA targeted towards MyD88 were custom designed with siRNA Invitrogen BLOCK-iT™ RNAi Designer.

The MyD88 siRNA sequences used are:

siRNA #1: 5'-UGGAGCAAACGGAGUCAAACUGAA-3'

siRNA #2: 5'-GAGUUUCCAAGUAUCUUGCGGUUCA-3'



siRNA #3: 5'-CAACUGUGUGGGUGUCCUACUUA-3'

A universal negative control siRNA (Stealth RNAi™ siRNA Negative Control LO GC), not homologous to anything in the vertebrate transcriptome and tested to not induce stress, was used to normalize relative gene inhibition of the target gene.

Twenty-four hours before transfection, 2.5 ml HD11 cells were transferred onto a 6-well plate (pre-plating) to reach 50 to 80% confluence, then were transfected with 100nm MyD88 siRNA S1, S2, S3, a combination of S1-3 or a nonsense negative control, using the chemical transfection reagent Lipofectamine RNAiMAX (Invitrogen). Transfection was performed according to the manufacturer's instructions. Briefly, siRNAs were diluted in 250 µl Opti-MEM medium and mixed gently. 5 µl Lipofectamine RNAiMAX was diluted in 250 µl Opti-MEM medium and combined with the diluted RNAi duplex, incubated for 20 min at room temperature, then added to each well containing cells to give a final volume of 3 ml and a final RNA concentration of 100 nm. The conditions for siRNA transfection were optimized by adjusting different siRNA concentrations (10, 50, and 100 nm) and different transfection methods (reverse and forward) using BLOCK-iT™ Alexa Fluor® Red Fluorescent Control (Invitrogen). Transfection efficiency was evaluated under a fluorescent microscope. Twenty four hours after transfection, HD11 cells were treated with 10 µg/ml and 0.0 µg/ml (non-treated) Type C GpG for 3 hours.

## **Results**

### **Dose and time effects on TLR15 expression after treatment with various TLR agonists in chicken HD11 cells**

TLR15 has been reported to be activated by heat-killed *Salmonella enterica* serovar Typhimurium infection at 48h post-injection in the cecum of 2 day-old chickens (Higgs *et al.*, 2006; Shaughnessy *et al.*, 2009). The specific stimulator of TLR15 is, however, unknown (Temperley *et al.*, 2008) and, therefore, we tested the responsiveness of TLR15 to various TLR agonists (Fig 1). To determine the appropriate time for harvesting cells following ligand induction, we quantified the expression level of TLR15 mRNA after induction with 0.0, 0.1, 1.0, 10.0 µg/ml of TLR stimulants at 3 and 24 hours of exposure (Table 1). TLR15 gene expression was higher in cells that were treated with 10 µg/ml *Salmonella enteritidis*-derived LPS than those of 0.1 µg/ml, 1 µg/ml and untreated cells ( $P = 0.0009$ ). TLR9 agonist CpG-ODN oligonucleotide (ODN) significantly induce TLR15 expression in cells treated with 10 µg/ml as compared to 0.1 µg/ml, 1 µg/ml and untreated cells ( $P = 0.03$ ). Addition of PAM3CSK4, peptidoglycan, E.coli derived LPS, CL075 thiazoloquinoline compound and poly I:C did not significantly induce TLR15 expression in HD11 cells.

Length of exposure had significant effects on TLR15 expression in HD11 cells treated with SE-LPS, CpG-ODN, CL075 and PAM3CSK4. TLR15 expression was significantly higher in cells at 3 hour post-stimulation (hps) with SE-LPS than at 24 hours ( $P = 0.003$ ). Exposure to CpG-ODN significantly induced TLR15 expression at 24 hps as compared to 3 hps ( $p < 0.0001$ ). The length of CL075 stimulation ( $P = 0.008$ ) and PAM3CSK4 stimulation ( $P = 0.0001$ ) significantly upregulated TLR15 expression in HD11 cells at 24 hps. Time and dose interaction was significant on HD11 cells treated with CpG-ODN ( $P = 0.04$ ). Because we are interested in the early TLR15 response to these stimulants in chicken macrophages, we

compared the expression level of TLR15 in stimulated and nonstimulated cells at 3 hps. Stimulation of HD11 cells with CpG-ODN, PAM3CSK4, *E.coli* K235 and SE derived LPS induced TLR15 expression at 3 hps (Table 2). However, we did not observe significant induction of TLR15 gene in thiazoloquinoline compound (CL075) and Poly I:C treated HD11 cells at this time of stimulation (Fig 1).

**TLR2, TLR4, IFNA and IL10 expression in CpG-ODN, PAM3CSK4, E.coli K235 and SE-derived LPS treated HD11 cells at 3 hours post-stimulation.**

TLR15, whose mRNA level increased following PAM3CSK4 (TLR2), *Salmonella* and *E.coli* derived LPS (TLR4) exposure, may be regulated by the stimulation of other TLRs and/or activation of proinflammatory cytokines. Hence, these receptors might be responding to similar stimulants. Interestingly, our findings showed that TLR15 was also significantly differentially expressed (DE) after stimulation with the TLR4 cognate ligand, LPS. IL10 and IFNA are produced through TLR9 in mammals in response to CpG-ODN stimulation (Takeshita *et al.*, 2004; Vollmer and Krieg, 2009). Therefore, we measured the expression of TLR2, TLR4, IFNA and IL10 genes in the same samples (Fig 2). Although TLR15 and TLR2 were both upregulated after *S. typhimurium* challenge in broilers (Higgs *et al.*, 2006), the response of TLR15 and TLR2 to CpG-ODN were significant ( $P = 0.03, 0.04$ , respectively) and antagonistic in HD11 cells in the present study, suggesting a possible cross-talk between TLR15 and TLR2 in the cellular membrane. IL10 ( $P = 0.04$ ) and IFNA ( $P = 0.03$ ) genes significantly downregulated in HD11 cells treated with CpG-ODN. However, we did not detect any significant change in the mRNA levels of IL10, IFNA and TLR2 in the

cells treated with PAM3CSK4, *E. coli* K235 and *S. enteritidis*-derived LPS at 3 hours post stimulation. TLR4 was not significantly differentially expressed in HD11 cells after stimulation with any of these ligands at 3 hours post stimulation (Table 3).

### **Stimulation of HD11 cells with A, B and C type CpG-ODNs**

We selected three types of ODNs that differ in their CpG motif and backbone composition and measured mRNA expression levels of TLR15, TLR21, TLR2, IFNA, IL1B and IL10 in HD11 cells (Fig 3). The TLR15 gene was significantly upregulated after B and C type CpG stimulated cells, compared to non-stimulated cells. In addition to TLR15, we quantified TLR21 mRNA levels because of the recent report as to its function as a mammalian TLR9 homologue. Interestingly, TLR21 expression was significantly down-regulated only after stimulation with type B ODN ( $P = 0.01$ ), a result that conflicts with the previously published data. The following genes were differentially expressed after treatment with the corresponding stimulant(s) ( $P < 0.05$ ): TLR2 (types B and C), IL1B (types B and C), IFNA (type C), IL6 (type C) and IL10 (types A and B) (Table 4). These results suggest that there is a high degree of similarity among the expression profile of TLR15, TLR2 and IL1B genes after CpG-ODN stimulation in HD11 cells at 3 hours post-stimulation.

### **Effect of CpG-ODN inhibitor (TLR9 antagonist) on TLR15 and MyD88 in HD11 cells**

Avian heterophils, monocytes, and macrophages can respond to CpG ODN in a manner similar to mammals, which results in increased proinflammatory cytokine production such as IL1B and IL6 (He *et al.*, 2003; Mackinnon *et al.*, 2009). After demonstrating the induction

of HD11 cells with CpG-ODN, we tested the effect of TLR9 inhibitor to further characterize the responsiveness to CpG-ODN in HD11 cells. We used an inhibitor ODN that competes with CpG-ODN and neutralize the stimulatory effect of CpG-ODNs. It acts by disrupting the co-localization of CpG-ODNs with mammalian TLR9 in endosomal vesicles without affecting cellular binding and uptake. The stimulation with this inhibitor is typically achieved with a 1:1-10 ratio of CpG-ODN: inhibitory ODN (Peter *et al.*, 2008). The differential expression levels of TLR15, TLR21, IL1B, IFNA, IL6 and IL10 genes were measured by QPCR after inhibitor ODN treatment in HD11 cells (Fig 4). TLR15 and IL1B genes were significantly down-regulated after macrophage treatment with the inhibitor ( $P < 0.05$ ). These same genes were significantly upregulated after CpG-ODN treatment at 3 hours post stimulation (Table 5). TLR21 was significantly downregulated after CpG-ODN induction - a result that supports the previous findings - and upregulated after treatment with the inhibitor ( $P < 0.05$ ). IFNA, IL6, IL10 and MyD88 genes were not consistently significantly differentially expressed after CpG-ODN or inhibitor ODN stimulation which suggest that IFNA, IL6, IL10 and MyD88 were unregulated in CpG-ODN induction assays.

#### **siRNA silencing of MyD88 gene affects IL1B expression after CpG-ODN induction**

To determine the MyD88 dependency of TLR15 to activate cellular immune response, we designed short interfering RNAs (siRNAs) directed at MyD88 gene and transfected HD11 cells with these siRNAs. Transfecting cells with the mixed siRNAs targeting MyD88 resulted in a 70% reduction in MyD88 mRNA expression (Fig 5). As shown in the previous experiments, C type CpG-ODN induction of HD11 cells resulted in significant upregulation

of IL1B expression along with TLR15 expression and downregulation of TLR21. In the control cells, with no reduction of MyD88 mRNA levels, CpG-ODN induction significantly upregulated IL1B. However, in the cells that had 70% less MyD88 transcripts, there was no significant differential expression of the IL1B gene (Table 6). This demonstrates that induction of IL1B expression via TLR15 signaling after CpG stimulation is MyD88-dependent. The development of an immune response through TLR15 requires MyD88 gene expression. TLR15, therefore, is not a gene like mammalian TLR4, which can activate both MyD88-dependent and MyD88-independent signaling pathways.

## Discussion

The determination of molecular mechanisms shaping chicken host response is of high priority for understanding the host-pathogen relationship and developing more effective disease control approaches in this major source of animal protein world-wide. We chose chicken macrophages HD11 cells as a model for their ability to generate a significant gene expression response to infectious reagents within hours. Also, the use of cell lines rather than primary cells provides functionally more uniform cells (Klasing, 1998).

Toll-like receptors are one of the gene families that have been strongly conserved over evolutionary time (Philbin *et al.*, 2005), and chicken TLRs are slightly different from other classes of organisms, based on the absence of TLR9 and the presence of TLR15, TLR1LA, TLR1LB, TLR21, and the pseudogene TLR8. Of the previously mentioned TLRs, fish (*Danio rerio*) and amphibia (*Xenopus laevis*) have both TLR21 and TLR9, while mammals only have TLR9. Despite the absence of chicken TLR9, chicken cells can still

response to CpG-ODNs and give rise to production of cytokines that are induced through TLR9 in mammals (Mackinnon *et al.*, 2009). Therefore, we hypothesized that the responsiveness of chicken cells to CpG-ODNs is dependent on the TLRs that do not exist in mammals. Although recent research revealed that TLR21 can respond to B type CpG-ODNs (Brownlie *et al.*, 2009), our findings suggest that a rapid response to multiple classes of CpG-ODNs in HD11 cells requires the differential expression of more than one TLR gene (Fig 6).

Multiple studies have shown differential expression of different TLR genes in response to CpG-ODNs. Nerren *et al.* (2010) showed upregulation of TLR15 in response to CpG-ODNs, while Brownlie *et al.* (2009) showed upregulation of TLR21 in response to CpG-ODNs (Brownlie *et al.*, 2009; Nerren *et al.*, 2009). Our findings suggest that these independent analyses may actually have observed the same phenomenon due to cross-regulation of TLR15 and TLR21. To further verify the downregulation of TLR21 and upregulation of TLR15 in response to CpG-ODN under our experimental conditions, cells were treated with CpG-ODN inhibitor. Our results showed significant upregulation of TLR21 and downregulation of TLR15, as expected. Moreover, different types of CpG-ODNs have distinct and in some cases contrasting effects on the same cells (Gursel *et al.*, 2002). Differences between our results and the literature reports might be due to dose (1.0 µg/ml vs 10.0 µg/ml), time periods (3, 24 h vs 0, 2, 4, 6, 8, 10 and 12 h) or other differences in experimental conditions (Brownlie *et al.*, 2009).

The present study demonstrated that TLR15 was rapidly upregulated in response to B and C type, but not to A type, CpG-ODNs (Fig. 6B, C) and, thus, it can distinguish the

different types of CpG-ODNs. Similarly, TLR2 and TLR21 respond differently to the different CpG-ODN types in chicken macrophage HD11 cells, suggesting that TLR15, TLR21, and TLR2 responses to CpG-ODNs are sequence-specific.

We suggest that there is a potential cross talk between TLR2 and TLR15 and, the end result of this cross talk may be induction of IL1B expression. It was previously shown that TLR2 and TLR15 were both upregulated in the cecum of *S. typhimurium* infected chickens and IL1B was significantly downregulated (Higgs *et al.*, 2006). Despite the differences in the direction of gene expression after CpG-ODN induction in the present study TLR2, TLR15, and IL1B were significantly differentially expressed after both B and C CpG-ODNs. Our findings on the CpG-ODN inhibitor effects on TLR15 and IL1B validate the IL1B induction through TLR15 (Fig 6A). After inhibitor treatment, TLR15 and IL1B were significantly co-downregulated.

A difference in MyD88 use is known to exist between mammals and chickens based on TLR4 responsiveness to LPS (Kestra and van Putten, 2008; Toshchakov *et al.*, 2002; Yamamoto *et al.*, 2003). The next question tested in the present study was about the mechanism of action of TLR15 gene after CpG-ODN induction. Is there a receptor in chickens that can activate both MyD88-dependent and –independent pathways? Because the treatment of HD11 cells with various stimulants such as Pam3Csk4, SE-LPS, EC-LPS and CpG-ODNs that contain PAMPs resulted in significantly differential expression of TLR15, the MyD88 use of this receptor was examined utilizing RNAi technology.



CpG-ODN recognition receptor TLR9 is already shown to operate with MyD88 dependent pathway in mammals (Bagchi *et al.*, 2007). Because TLR9 is not present in avian species, it is not known whether the functional orthologs of TLR9 in chickens utilize the same adaptor molecule. Our findings demonstrated that both TLR15 and TLR21 use a MyD88-dependent pathway to regulate IL1B gene expression in HD11 cells as does mammalian TLR9. TLR15 and TLR21 were significantly differentially expressed in HD11 cells after stimulation, regardless of siRNA transfection. However, IL1B was significantly differentially expressed in all cells, except those with siRNA-induced knockdown of MyD88 gene expression (Fig 6D). A decrease in MyD88 expression of 70% was achieved when the cells were simultaneously transfected with the three siRNAs targeting MyD88, but no decrease was observed in cells transfected with individual siRNAs. Similarly, IL1B was significantly differentially expressed in those cells which were transfected with individual siRNAs. Additionally, because the insertion of nucleic acid fragments into cells might give rise to an immune response, siRNAs targeting MyD88 gene were designed considering the potential base motifs to avoid unintended cellular response. MyD88 expression was not significantly changed in response to CpG-ODNs or CpG-ODN inhibitor in non-transfected cells, similar to results showing ubiquitous expression of MyD88 in specific human tissues (Pioli *et al.*, 2004). This result indicated that the TLRs are not regulating IL1B through differential expression of MyD88, but likely only through use of MyD88 as a signaling molecule. Accordingly, although MyD88 is not transcriptionally regulated, the amount of MyD88 protein in the cytosol, at any given time, is important for the production of

proinflammatory cytokines after CpG-ODN induction. Future research should determine whether other chicken-specific TLRs utilize MyD88-dependent and/or –independent pathways in response to CpG-ODN or other stimulants,

Our findings characterized the regulation and the mechanism of action of chicken macrophage host response after stimulation with CpG-ODNs and the data clearly demonstrate that engagement of the MyD88 dependent pathway with TLR agonist CpG-ODNs in chickens influences host inflammatory responses in infections. The CpG-ODN immune response pathway in chicken macrophage cells is similar to the pathway in mammals, in that both are MyD88-dependent, thereby pointing to the conservation of this mechanism over long evolutionary distance of mammals and aves.

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## TABLES

Table 1. TLR agonist dose and exposure time effect on TLR15 mRNA levels in chicken macrophage HD11 cells; Expression levels of mRNA for each gene were analyzed by using JMP software with ANOVA model were considered fixed main effects. The interactions for the fixed main effects were also tested for P value calculation.

<u>Stimulant</u>	<u>Putative Receptor</u>	<u>Time</u>	<u>Dose</u>	<u>Time*Dose</u>
CpG-ODN	TLR9	<0.000	0.03	0.04
SE-LPS	TLR4	0.006	0.002	0.29
Pam3Csk4	TLR2	<0.000	0.09	0.19
EC-LPS	TLR4	0.99	0.16	0.47
Poly I:C	TLR3	0.78	0.14	0.92
CL075	TLR7/8	0.02	0.41	0.49
SA-PGN	TLR2	0.82	0.33	0.70

*P* values obtained from ANOVA



Table 2. TLR agonist dose [0.0 (control), 0.1, 1.0, 10.0 µg/ml] effect on TLR15 mRNA levels in chicken macrophage (HD11) cells at 3 hps. *P* values by ANOVA.

<u>Stimulant</u>	<u>TLR15</u>
C type CpG	0.026
SE-LPS	0.052
Pam3Csk4	0.022
EC-LPS	0.002
Poly-IC	0.472
CL075	0.748
SA-PGN	0.841

Table 3. TLR agonist effects on the expression of cytokines that are produced via TLR9 signaling and on the expression of TLR2 and TLR4.

<u>Stimulant</u>	<u>Receptor</u>	<u>IFNA</u>	<u>IL10</u>	<u>TLR2</u>	<u>TLR4</u>
E-LPS	TLR4	0.88	0.41	0.20	0.37
PAM3CSK4	TLR2	0.68	0.77	0.58	0.58
SE-LPS	TLR4	0.80	0.10	0.79	0.70
CpG	TLR9	0.03	0.04	0.04	0.16

Table 4. Effect of different types of CpG-ODNs on TLR15, TLR21, IL1B , IFNA , IL10 and TLR2 in chicken macrophage (HD11) cells at 3 hps. *P* values calculated by contrast analysis; stimulated vs non-stimulated. TLR15 C(T) values were normalized to 28 rRNA reference gene.

<u>CpG Types</u>	<u>TLR15</u>	<u>TLR21</u>	<u>IL1B</u>	<u>IFNA</u>	<u>IL10</u>	<u>TLR2</u>
A (1585)	0.130	0.60	0.570	0.28	0.02	0.236
B (1668)	0.004	0.03	0.000	0.80	0.03	0.003
C(2395)	0.003	0.19	0.001	0.04	0.72	0.061

Table 5. Effect of CpG-ODN inhibitor on the selected genes' expression in chicken macrophage (HD11) cells at 3 hps. *P* values obtained from contrast analysis. (1U: 10µg/ml)

<u>CpG-ODN vs CpG-ODN+Inhibitor</u>	<u>TLR15</u>	<u>TLR21</u>	<u>IL1B</u>	<u>IFNA</u>	<u>IL10</u>	<u>IL6</u>	<u>MyD88</u>
1U vs 1U +1U	0.08	0.50	0.023	0.73	0.94	0.09	0.69
1U vs 1U +5U	0.02	0.39	0.015	0.58	0.36	0.24	0.65
1U vs 1U +10U	0.76	0.08	0.083	0.62	0.63	0.86	0.43
CpG stimulated vs non-stimulated	0.0001	0.57	0.009	0.60	0.15	0.002	0.88

Table 6. Effect of siRNAs targeting chicken MyD88 gene on IL1B expression in chicken macrophage (HD11) cells stimulated with C type CpG-ODN P values were obtained from contrast analysis.

<u>siRNA (CpG)</u>	<u>MyD88</u>	<u>IL1B</u>	<u>CpG-ODN</u>	<u>TLR15</u>	<u>TLR21</u>
NEG(-) vs S1(-)	0.70	0.28	(+) vs (-)	0.001	0.07
NEG(-) vs S2(-)	0.75	0.55			
NEG(-) vs S3(-)	0.20	0.26			
NEG(-) vs MIX(-)	0.21	0.57			
NEG(-) vs S1(+)	0.42	0.60			
NEG(-) vs S2(+)	0.81	0.17			
NEG(-) vs S3(+)	0.84	0.06			
NEG(-) vs MIX(+)	0.03	0.42			

## FIGURES

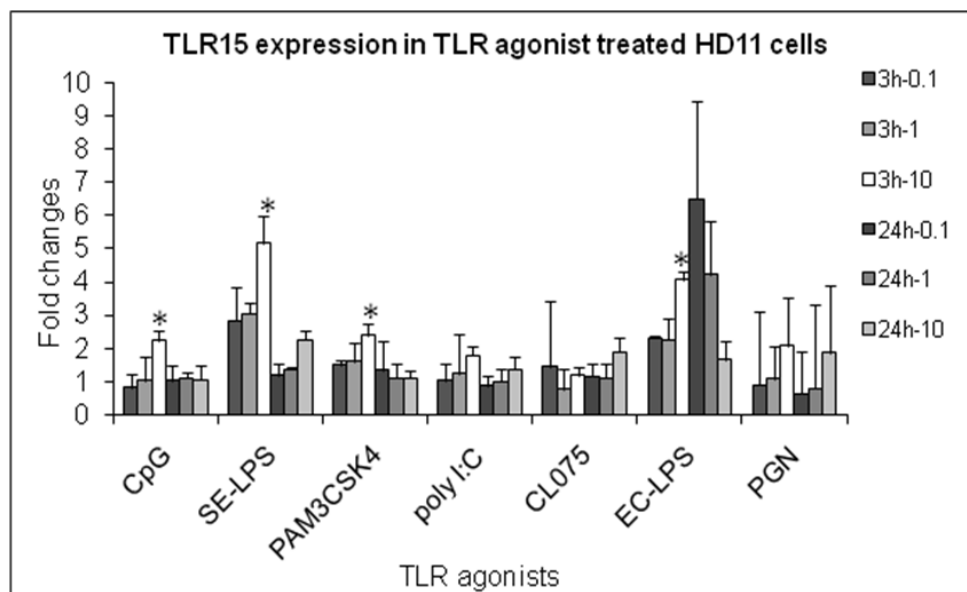


Figure 1. TLR15 expression after 3 and 24 hours of stimulation with CpG-ODN, SE-LPS, PAM3CSK4, Poly (I:C), CL075, Peptidoglycan. Doses: 0.0 (nonstimulated), 0.1, 1.0, 10.0 µg/ml. (\* significant at  $P < 0.05$ ; stimulated vs non stimulated).

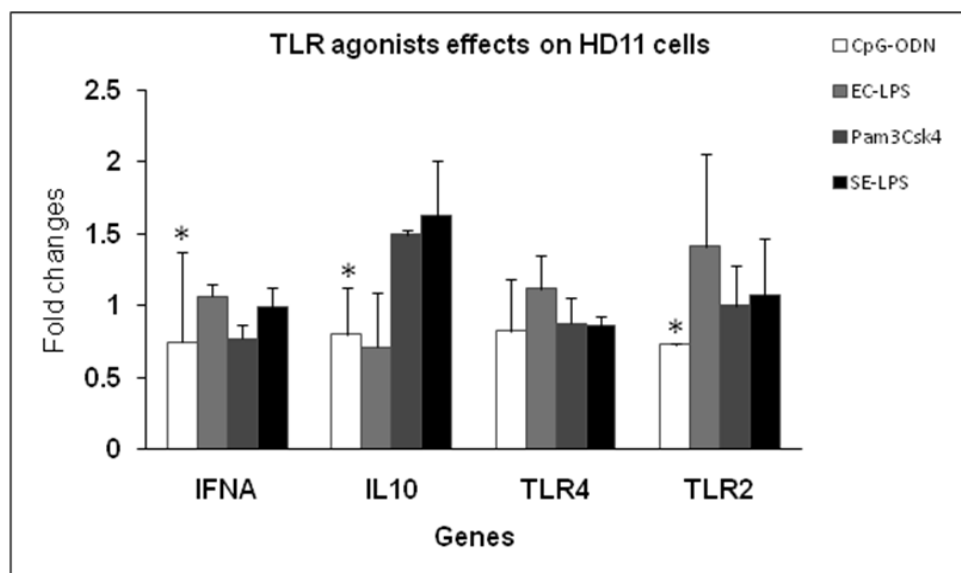


Figure 2. IFNA , IL10, TLR4 and TLR2 expression (fold changes) in chicken macrophage (HD11) cells stimulated with CpG-ODN, LPS from E.coli and S. enteritidis, PAM3CK4. (\* significant at  $P < 0.05$ ; stimulated vs nonstimulated).

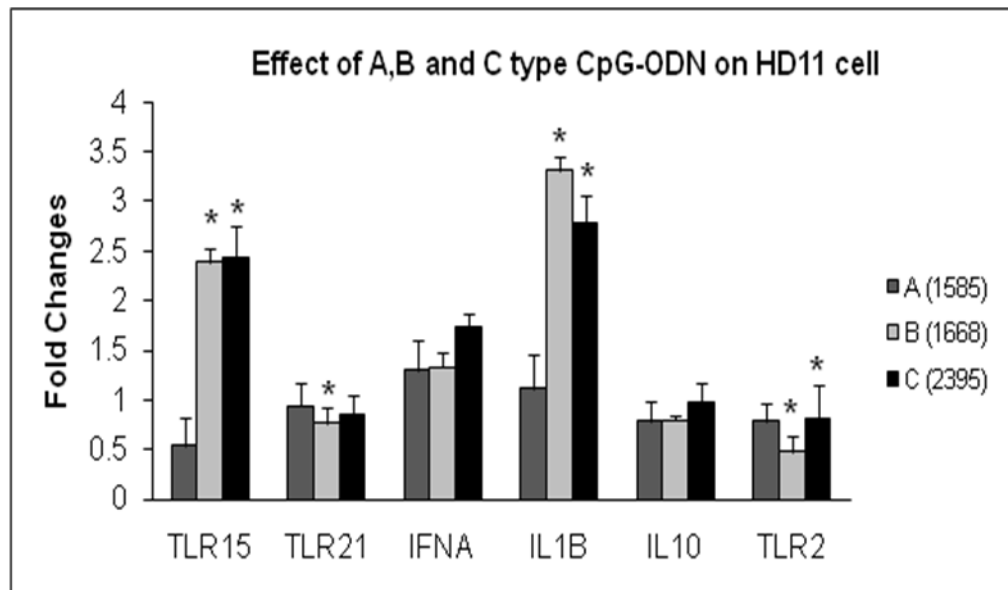


Figure 3. TLR15, TLR21, IFNA , IL1B and IL10 fold changes in A, B and C type CpG-ODN induced chicken macrophage (HD11) cells at 3 hps. \* Significant at  $P < 0.05$ ; contrast analysis of LS means of C(t) values shows the significant difference between stimulated and non stimulated cells.



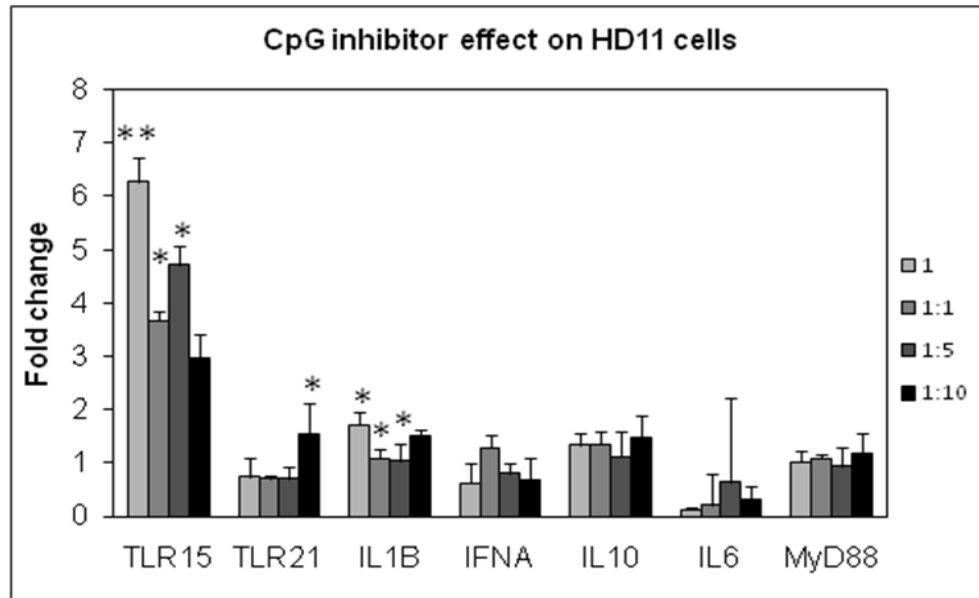


Figure 4. Effect of CpG-ODN inhibitor on TLR15, TLR21, IL1B , IFNA , IL10, IL6 and MyD88 expression in chicken macrophage (HD11) cells. Cell culture conditions (1): 10µg/ml CpG-ODN; (1:1): 10µg/ml CpG-ODN+10µg/ml inh ODN; (1:5): 10µg/ml CpG-ODN+50µg/ml inh ODN; (1:10): 10µg/ml CpG-ODN+100µg/ml inh ODN. \* Significant  $P < 0.05$ ; contrast analysis of LS means of C(t) values shows the significant difference between CpG-ODN treated cells and CpG-ODN+inhibitor ODN treated cells. \*\* Significant at  $P < 0.01$ ; contrast analysis shows the significant difference between CpG-ODN stimulated and non stimulated cells.

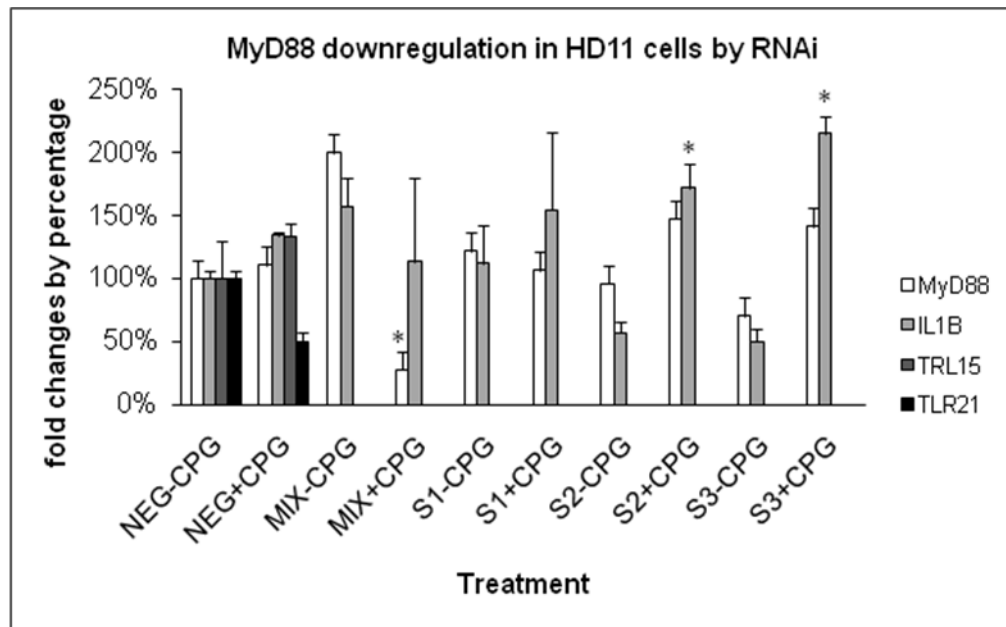


Figure 5. RNAi mediates downregulation of chicken MyD88 gene. chicken macrophage (HD11) cells were transfected with negative siRNA or 3 different siRNA targeting MyD88 (S1, S2, S3) or co-transfected with the combination of S1, S2 and S3 siRNAs. 24 hours after transfection, HD11 cells were treated with C type CpG-ODN and cell were harvested following 3 hours stimulation and analyzed by QPCR. TLR15, TLR21 and IL1B genes expression were measured by QPCR. Data are presented as percentages compared with the negative siRNA. Experiments were carried out in triplicate. \* indicates  $P < 0.05$ ; analysis by contrast analysis of LS means of C(t) values shows the significant difference between negative siRNA transfected and siRNA transfected. IL1B was upregulated in cell where siRNAs did not reduce MyD88 expression.

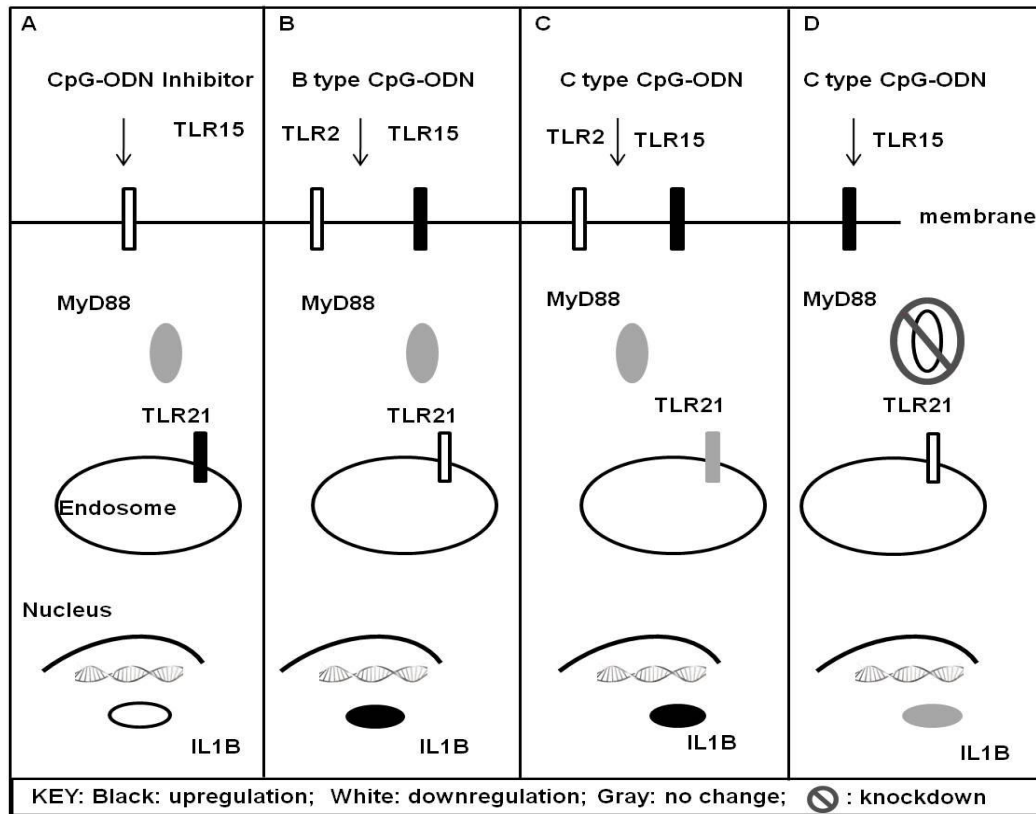


Figure 6. Schematic diagram of the regulation and mechanism of action of CpG-ODN response in chicken macrophage (HD11) cells.

## **Chapter 3: Unique genome-wide transcriptome profiles of chicken macrophages exposed to *Salmonella*-derived endotoxin**

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### **Abstract**

#### **Background**

Macrophages play essential roles in both innate and adaptive immune responses. Bacteria require endotoxin, a complex lipopolysaccharide, for outer membrane permeability and the host interprets endotoxin as a signal to initiate an innate immune response. The focus of this study is kinetic and global transcriptional analysis of the chicken macrophage response to *in vitro* stimulation with endotoxin from *Salmonella typhimurium*-798.

#### **Results**

The 38535-probeset Affymetrix GeneChip Chicken Genome array was used to profile transcriptional response to endotoxin 1, 2, 4, and 8 hours post stimulation (hps). Using a maximum FDR (False Discovery Rate) of 0.05 to declare genes as differentially expressed (DE), we found 13, 33, 1761 and 61 DE genes between endotoxin-stimulated versus non-stimulated cells at 1, 2, 4 and 8 hps, respectively. QPCR demonstrated that endotoxin

exposure significantly affected the mRNA expression of *IL1B*, *IL6*, *IL8*, and *TLR15*, but not *IL10* and *IFNG* in HD 11 cells. Ingenuity Pathway Analysis showed that 10% of the total DE genes were involved in inflammatory response. Three, 9.7, 96.8, and 11.8 % of the total DE inflammatory response genes were significantly differentially expressed with endotoxin stimulation at 1, 2, 4 and 8 hps, respectively. The *NFKBIA*, *IL1B*, *IL8* and *CCL4* genes were consistently induced at all times after endotoxin treatment. *NLRC5* (CARD domain containing, NOD-like receptor family, RCJMB04\_18i2), an intracellular receptor, was induced in HD11 cells treated with endotoxin.

## Conclusions

As above using an *in vitro* model of chicken response to endotoxin, our data revealed the kinetics of gene networks involved in host response to endotoxin and extend the known complexity of networks in chicken immune response to Gram-negative bacteria such as *Salmonella*. The induction of *NFKBIA*, *IL1B*, *IL8*, *CCL4* genes is a consistent signature of host response to endotoxin over time. We make the first report of induction of a NOD-like receptor family member in response to *Salmonella* endotoxin in chicken macrophages.

## Background

Determining the effects of endotoxin from *Salmonella typhimurium* in chicken macrophages is an *in vitro* model to characterize the transcription profiles of one important cell type in the chickens' immune response. Endotoxin is a complex lipopolysaccharide (LPS) found in the outer cell membrane of Gram-negative bacteria that is responsible for

membrane organization and stability (Gorbet and Sefton, 2005) and differs from LPS in that it is a butanol/water extract rather than a phenol/water extract (Beutler and Rietschel, 2003). Endotoxin used in the present study is between 10 and 20% protein and reproducible, hence its complexity better mimics the cell membrane *in vivo*. Recognition of the lipid A and/or the polysaccharide moiety of endotoxin by membrane receptors of monocytes induce a wide variety of cellular responses, including the synthesis of cytokines such as *IL1B*, *TNF*, *IL6*, *IL8* (Agarwal *et al.*, 1995). Vertebrates have evolved an effective innate immune response to LPS-containing bacteria over evolutionary time. Chickens are much more resistant than mammals to LPS-induced septic shock (Kestra and van Putten, 2008) and respond to LPS with the induction of *IL1B*, *IL6*, and *IL18* mRNA (Kogut *et al.*, 2006). However, few studies have specifically examined the response to the more complex and more relevant immune stimulant, endotoxin, as a model for *in vivo* responses.

Membrane-bound receptors (some Toll-like Receptors; TLRs) and also intracellular receptors such as NOD-like Receptors (NLRs) play key roles in the recognition of pathogen associated molecular patterns (PAMPs) to induce a host response. Both receptor families contain a series of Leucine Rich Repeat (LRR) modules in their ligand recognition domains (Monie *et al.*, 2009). Although NLRs have been extensively studied in mammals (Totemeyer *et al.*, 2006), their regulation in chicken is still to be described

Macrophages play primary roles in both innate and adaptive immunity. In addition to their roles in innate disease resistance, macrophages are versatile cells that can alter the animal's immunological state by producing regulatory molecules such as cytokines,

enzymes, and receptors that regulate the adaptive immune response (Qureshi *et al.*, 2000). Cell lines allow better experimental control and reproducibility than primary cultures of macrophages because of the functional uniformity of cell populations (Klasing, 1998). Despite the limited number of studies with chicken macrophages, it is known that they are capable of mediating lymphoid functions (Qureshi, 1998). HD11 is an avian myelocytomatosis virus (MC29) transformed chicken macrophage-like cell line (Beug *et al.*, 1979) that has been extensively studied. For example, LPS induced a significant level of nitric oxide production (NO) in HD11 cells (Dil and Qureshi, 2002). HD11 cells have been shown to be activated, as measured by NO production, by various doses of LPS by He *et al.* (2006) (He *et al.*, 2006). This dose-dependent induction of NO in HD11 cells at 24 hours post stimulation demonstrates involvement in host response mechanisms to microbial infections and responsiveness of HD11 cells to bacterial components.

Gene expression profiling using microarrays is a widely used method to explore biological functions of both host and microorganisms in innate immunity (Jenner and Young, 2005; Zhang *et al.*, 2009). Classifying interconnected and overlapping components of the immune system into subsets, according to their functionality, such as cellular versus humoral immunity or innate versus adaptive immunity, permit the complex immune system to be dissected into distinct areas. Chicken macrophage immune response to strains of avian pathogenic *Escherichia coli* (APEC) and *Mycoplasma synoviae* was previously studied in HD11 cells using the avian macrophage microarray (AMM) with 4906 elements and using the avian innate immunity microarray (AIIM) with 4959 elements (Lavric *et al.*, 2008). The

AMM with 4906 elements has also been used by Bliss *et al.* (2005) to determine the avian macrophage response to commercial *Salmonella typhimurium* lipopolysaccharide (Bliss *et al.*, 2005). However, the AMM profiling tool lacked some important elements; for example, replicates of probes for known Toll-like receptor genes were missing. Transcriptional profiling of chicken HD11 cells stimulated with *Salmonella enteritidis* was performed using the AMM array, and the authors reported that most of the DE genes responded at 5 hours post stimulation, with more genes down-regulated than up-regulated (Zhang *et al.*, 2008).

In the present study, a global transcriptome analysis of the HD11 innate immune response was conducted. The HD11 cells were exposed to various doses of ST-798 endotoxin for 1, 2, 4, and 8 hours and the mRNA levels of *IL6*, *IL8*, *IL10*, *IL1B*, *IFNG*, and *TLR15* genes were measured by Quantitative RT-PCR and with the *Affymetrix* GeneChip containing 38535 probes. First, we determined the optimum among four endotoxin doses to elicit an immune response in HD11 cells and then performed a microarray experiment. Our results showed a chicken host response to *Salmonella* endotoxin that initiated quickly and significantly, increased in breadth up to 4 hps, and then rapidly approached homeostasis at 8 hps. The data suggest the importance of these early-induced genes in initiating the extensive gene cascade occurring at 4 hours exposure. We classified all significantly differentially expressed genes by their function and compared gene networks at 1, 2, 4, and 8 hours post-stimulation. The large number of genes differentially expressed at 4 hours enabled the elucidation of highly refined gene networks. This study provides a more comprehensive assessment of chicken macrophage response to endotoxin from *Salmonella typhimurium* (one



of the most common food-borne pathogen) than the literature published to date, along with other novel findings on specific genes

## Results

### Endotoxin dose of 1 µg/ml consistently induces an immune response in chicken macrophages

HD11 cells were stimulated with 0.0, 0.1, 1.0, or 10.0 µg/ml endotoxin for 1, 2, 4, or 8 hours and the differential expression of *IL6*, *IL8*, *IL10*, *IL1B*, *IFNG*, and *TLR15* genes was measured by QPCR. Multiple comparison analysis of least squares means (LSmeans) demonstrated that 1 µg/ml of endotoxin was the minimum concentration required to elicit an immune response in HD11 cells, assayed by transcriptional differences in these selected genes (Table 1). Macrophages stimulated with endotoxin expressed significantly higher levels of *IL6*, *IL8*, *IL1B*, and *TLR15* than the non-stimulated (vehicle-treated) macrophages. Cells stimulated with 1.0 µg/ml endotoxin also expressed higher mRNA than cells stimulated with 0.1 µg/ml endotoxin for *IL1B* ( $P < 0.0001$ ) and *IL6* ( $P = 0.03$ ). Stimulation of cells with endotoxin of all doses induced higher *IL8* expression than in non-stimulated cells ( $P < 0.0001$ ). Cells stimulated with 1.0 µg/ml endotoxin expressed higher levels of *TLR15* than non-stimulated cells ( $P = 0.002$ ). *IL10* gene expression did not change by endotoxin dose. The stimulation time had significant effect on the mRNA levels of all genes assayed by QPCR. The endotoxin dose significantly affected the expression of *TLR15*, *IL1B*, *IL8* and *IL6* (Table 2). Thus, endotoxin stimulation of HD11 macrophages had different impacts on

each gene (Table 3). *IL1B*, *IL8*, *IL6* and *TLR15* gene expression differed by the endotoxin dose, while no *IFNG* or *IL10* induction was measured after endotoxin stimulation (Fig. 1A). Endotoxin treatment, comparing treated to non-treated cells, had significant or near significant effects on *IL1B* and *IL8* genes at 2, 4 and 8 hps (Fig. 1B, 1C, 1D).

### **Transcriptional response of chicken macrophages to *Salmonella* endotoxin**

We used the array to profile the transcriptional response of chicken HD11 cells to endotoxin over time. We found 13, 33, 1761, and 61 genes significantly DE between endotoxin-stimulated (1.0 µg/ml) and vehicle-treated HD11 cells at 1, 2, 4, and 8 hours; respectively ( $q < 0.05$ ). Our results provide a unique and more comprehensive chicken transcriptome profile than current literature.

Comparative analysis of DE genes by Ingenuity Pathway Analysis showed that 10% of the total DE genes are annotated as inflammatory response. Three, 9.7, 96.8, and 11.8 % of these inflammatory response genes were significantly affected at 1, 2, 4, and 8 hours, respectively [additional file 1].

The 13 genes responding to endotoxin stimulus at 1 hour exposure were *TNFAIP3* (Tumor Necrosis Factor, alpha-induced protein 3) [GeneBank: XR\_026935], *TNIP2* (TNFAIP3 interacting protein 2) [GeneBank: NM\_001031166], *NFKBIA* (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) [GeneBank: NM\_001001472], *MRGPRH* (seven transmembrane domain G-protein coupled receptor) [GeneBank: XM\_418053], *BTG2* (B-cell translocation gene 2) [GeneBank: XM\_418053],

*IL1B* (Interleukin 1 Beta) [GeneBank:NM\_204524], *CCL4* (chemokine (C-C motif) ligand 4) [GeneBank: NM\_001030360], *CD83* (CD83 antigen, activated B cells) [GeneBank: XM\_418929], *IL8* (Interleukin 8) [GeneBank: NM\_205498], *CH25H* (cholesterol 25-hydroxylase) [GeneBank: XM\_421660], *TRAF3* (TNF receptor-associated factor 3) [GeneBank:XM\_421378], and *JUN* (jun oncogene) [GeneBank: NM\_001031289] genes.

Most, if not all, of these 13 genes have key roles in the immune response and were significantly up-regulated (Table 4). The number of significantly DE genes increased from 13 to 33 at 2 hours post stimulation. Four hours after stimulation, 1761 genes were differentially expressed with about 2/3 up-regulated and 1/3 down-regulated [additional file 2]. Interestingly, all stimulated genes at both 1 hps and 2 hps except *LIPG* were still up-regulated at 4 hps, and all but three (*TRAF3*, *JUN* and *TNIP2*) of the genes stimulated at 1 hps, and all but 4 of the 2 hps up-regulated genes (*DUSP10* and three un-annotated transcripts), remained elevated at 8 hps, indicating much of the earliest immune response stimulation was still occurring. Clearly, however, the majority of the massive response observed at 4 hps was very transitory, significantly shutting down by 8 hps.

### **Persistent inflammatory response across all time points but a specific anti-microbial response only at 4 hours after endotoxin stimulation**

Transcriptional regulation of chicken macrophages changed as a result of endotoxin treatment observed as early as 1 hour post exposure. To explore these changes, we categorized DE genes by function, with an emphasis on immunological functions, and compared the *P*-values for all time points within each functional group using Ingenuity

Pathway Analysis (IPA) software (Fig. 2). The significance levels varied across the functional groups. Genes annotated with various types of immune and inflammatory response functions were significantly overrepresented in all gene lists. However, the genes in the “Antimicrobial Response” functional category were differentially expressed only at 4 hps, demonstrating the specific character of the immune response of chicken macrophages to ST-798 endotoxin at 4 hps.

### **Genes involved in “immune cell trafficking” networks after endotoxin stimulation**

We then used IPA for comparative gene network analysis (Fig. 3, 4, 5). Ingenuity Pathway Analysis considers all possible interactions between the genes, including the ones that are not in the entered gene list. During the first hour of endotoxin exposure, only 13 genes were significantly up-regulated, which resulted in a network only lightly populated with our DE genes and thus provided little insight (Fig. 3, 5).

The one hour post-stimulation response was the limiting factor in network comparisons because of the small number of differentially expressed genes. Gene networks of “immune cell trafficking” were identifiable at all four time points, however, and therefore were used for comparison of network structure over time. At 1 hps, the *BTG2*, *IL8*, *TNIP2* and *CCL4* genes were included in the “Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking” group according to their function [additional file 3]. *NFKBIA*, *IL1B*, *IL8*, and *CCL4* genes were persistently up-regulated at each time point. *API* (*JUN*) transcription factor was induced

when macrophages were exposed to endotoxin for 1 hour, but this expression profile was not observed at 8 hours exposure. However, an *NFKB* dependent host response was shown by the significant differential expression of *NFKBIA*. Phosphorylation and the subsequent ubiquitination of IKB, the gene product of the *NFKBIA* gene, are known as key processes required for regulating the innate immune system (Bhoj and Chen, 2009). We observed a significant increase, after endotoxin stimulation at 4 hours, in the mRNA levels of IL-1 receptor-associated kinase 2 (*IRAK2*) which regulates phosphorylation and the genes that are involved in ubiquitination: ubiquitin-conjugating enzyme E2Q family member 2 (*UBE2Q2*), ubiquitin protein ligase E3C (*UBE3C*), ubiquitin-conjugating enzyme E2A (RAD6 homolog) (*UBE2A*), ubiquitin-conjugating enzyme E2, J1 (UBC6 homolog, yeast) (*UBE2J1*), ubiquitination factor E4B (UFD2 homolog, yeast) (*UBE4B*) (Table 5, additional file 2). Because the number of differentially expressed genes increased with time up to 4 hps, we were able to define more precise interactions at that time among the analyzed genes using the Ingenuity Pathway Analysis software (Fig. 4) IL1 receptor family members, *IL1RL2* (interleukin 1 receptor-like 2) and *IL1R2* (interleukin 1 receptor, beta) were responsive to 4 hours of endotoxin stimulation relative to untreated cells (fold change = 1.4, 2.2;  $q = 0.039811$ , 0.001; respectively). We conclude that *IL1B* is a central node in the cellular response network due to its coordination and interactions with other molecules in the network (Fig. 4). The functions of all genes demonstrated in the networks at all time points are indicated in additional file 2.

### **The differential expression of receptors in HD11 cells upon exposure to endotoxin**

Fold changes in the DE genes ranged from  $1.68^{-1}$  to 5.65 at all time points, but  $q$  values were highly significant ( $q = 0.05$ ) [additional file 2]. We did not detect a significant increase in mRNA level of any *TLR* during the course of exposure; however, *TLR2* was significantly down-regulated at 4 hps (fold change,  $1.65^{-1}$ ;  $q = 0.009756$ ) (Fig. 6).

Interestingly, *NLRC5* (CARD domain containing, NLR family, RCJMB04\_18i2), an intracellular receptor, in HD11 cells treated with endotoxin for 4 hours (fold change, 1.4;  $q = 0.047$ ) was induced in the present study. Similar to *TLRs*, the *NLRs* recognize pathogen associated molecular patterns that are expressed by bacteria and then activate translocation of NF $\kappa$ B from the cytosol to the nucleus. *NLRC5* was responsive to endotoxin; however it was not included in either gene networks or functional groups [additional file 1]. Despite accumulating research data, the exact molecular mechanism of *NLR* activation and the initiation of signaling cascades in mammals are not yet fully defined (Proell *et al.*, 2008). The data of the present study, however, clearly identify a role for *NLRC5* in chicken macrophage response to endotoxin.

## Discussion

We did not detect any significant up-regulation in the mRNA levels of *TLR3*, *TLR4*, *TLR5*, *TLR6*, *TLR7*, *TLR15*, *LOC768669* (similar to *TLR1*)/*TLR16* / *TLR6* (eukaryotic homology group) in the microarray results of this study. Only *TLR2* showed a significant change in the mRNA level and was slightly, but significantly, down-regulated in stimulated cells. In contrast, *NLRC5* (NLR family, CARD domain containing 5), was significantly up-regulated. The downregulation of *TLR2* might be considered as a result of *NLRC5* activation

after endotoxin stimulation. The inhibitory effects of NLRC5 on inflammatory pathways have recently been reported (Benko *et al.*, 2010).

Chicken Tumor Necrosis Factor (*TNF*) alpha gene has not been identified in the chicken genome yet. Interestingly, our study reports differential expression of three TNFalpha-related genes after 1 hour endotoxin exposure, including *TNFAIP3* (Tumor Necrosis Factor, alpha-induced protein 3), *TNIP2* (TNFAIP3 interacting protein 2), and *TRAF3* (TNF receptor-associated factor 3) genes, thus providing additional evidence of existence of genes with TNFA function in chickens. There are still numerous cytokines to be identified, because of limitations in the completeness of the chicken genome assembly (Kaiser *et al.*, 2005).

Inflammatory response to infections and tissue injuries is a complex process. Because the inflammatory response causes tissue damage and significant changes in tissue physiology, it must be tightly regulated. The genes that encode antimicrobial effectors do not cause tissue damage and are important for the macrophage early host defence (Foster *et al.*, 2007). The differential expression of antimicrobial effectors, but not other functional categories at 4 hps, may be an indication of a self-tolerance mechanism that was developed by chicken macrophages.

Mammals and birds diverged 300 million years ago. There are evolutionarily conserved regions on the chromosomes of both classes (Burt *et al.*, 1995) such as Toll-like receptor (TLR) encoding genes (Philbin *et al.*, 2005). Specific receptor for LPS is TLR4 in

mammals. It can make the combined use of MyD88-dependent and –independent signalling pathway, while chicken TLR4 cannot. Key components involved in mammalian MyD88-independent TLR4 signalling are LPS Binding Protein (LBP), the lipid scavenger protein CD14, and the intracellular adaptor molecule TRAM (Jiang *et al.*, 2005). Examination of the chicken genome demonstrates no orthologs for these proteins, with the exception of a CD14-like molecule.

Based on the similarities among the experimental designs, we compared our findings with those reported by Bliss *et al.*, (2005) and Zhang *et al.*, (2008) using the NCBI GenBank gene expression omnibus (GEO) repository, series accession number [GSE1794] (Bliss *et al.*, 2005; Zhang *et al.*, 2008). Our comparison included inflammatory response genes which were classified by Ingenuity Pathway Analysis software. *IL1B* and *IL8* genes were the only genes that showed upregulation in all three studies. Zhang *et al.*, (2008) expression data reported upregulations for *CCL4* and *CD83* genes, while our results were in concordance with Bliss *et al.*, (2005) on the expressions of *TRAF6*, *c-fos*, and *TLR1/16/6* genes (Bliss *et al.*, 2005; Zhang *et al.*, 2008). The rest of the compared genes did not show a commonality in the expression, probably due to the differences among the experimental conditions, exposure time and the stimulator.

One of the promoter regulatory elements that mediates LPS response in human monocytes is the TPA (12-O-tetradecanoylphorbol 13-acetate)-response element (TRE). The transcription factors that bind to TRE sites are called the Activator Protein 1 (AP-1) complex. They are composed of both the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fral, and



Fra2) families (Mackman *et al.*, 1991). AP-1 activity is regulated by induced transcription of *c-Fos* and *c-Jun* and/or by posttranslational modification of their products in mammals. c-Jun is ubiquitously present in cells in an inactive form that can be activated through phosphorylation by c-Jun N-terminal kinase (JNK), which belongs to the MAP kinase family (Karin and Hunter, 1995). Kogut *et al.*, (2008) demonstrated that chicken heterophils stimulated with flagellin and LPS exhibited a significant increase in DNA binding by the AP-1 family members c-Jun and JunD (Kogut *et al.*, 2008). The current study shows significant induction of *MAPK8* at 4 hps (1.32 fold;  $q < 0.04$ ) that may have activated *JUN* at 4 hps. Exposure of cells to various stimulants results in the release of NF $\kappa$ B from inhibitor I $\kappa$ B that controls NF $\kappa$ B activity. Signals activate NF $\kappa$ B by targeting I $\kappa$ B for proteolysis (Alkalay *et al.*, 1995). I $\kappa$ B is degraded by a phosphorylation-dependent ubiquitination process. Our data report the significant up-regulation of *IRAK2* gene and the genes that are involved in the ubiquitination process to activate NF $\kappa$ B (Table 5).

Although QPCR results showed higher fold changes than the microarray data, they supported the microarray data as to direction of change for the majority of the genes tested. QPCR is able to measure much larger expression changes than microarray because of the larger dynamic range of QPCR experiments (Baum *et al.*, 2003). Moreover, the two methods require and use different normalization methods (Morey *et al.*, 2006).

## Conclusions

We investigated the transcriptional response after in vitro exposure to endotoxin from *Salmonella typhimurium*-798 of the chicken macrophage cell line HD11 as a model for chicken host response to bacteria. Both QPCR and microarray analysis were performed to define the magnitude and the kinetics of innate immune response. Our data showed a strong macrophage response to endotoxin at 4 h post-stimulation, which decreased dramatically by 8 h post-stimulation. About two-thirds of the significantly differentially expressed genes were up-regulated. The *NFKBIA*, *IL1B*, *IL8*, and *CCL4* genes were consistently induced at all time points after endotoxin treatment, demonstrating their important role in response to *Salmonella*. Additionally, the up-regulation of *JUN* and *MAPK8* at 4 h post-stimulation shows chicken cells use this additional pathway to induce an immune response through the AP1 transcription factor. Although none of the TLRs were upregulated after endotoxin stimulation, the CARD5 domain containing NOD like Receptor 5 (*NLRC5*), an intracellular receptor, was upregulated in response to *Salmonella* endotoxin. To our knowledge, this is the first report of the *NLRC5* induction by bacterial membrane components in chickens. The recognition of *Salmonella typhimurium*-798 endotoxin by chicken macrophages clearly caused multiple signalling cascades to be initiated and resulted in many gene expression changes. The number of DE genes decreased by 96% from 4 hours to 8 hours post stimulation. This suggests that chicken macrophages quickly return to homeostasis after response to endotoxin-caused shock. This study enhances knowledge on the chicken

macrophage transcriptional response to endotoxin by elucidating the complex gene networks involved in the chicken inflammatory response and reports the novel involvement of *NLRC5*.

## **Methods**

### **Cell Culture and Stimulation**

The chicken HD11 macrophage cell line [11] was cultured in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated newborn calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES and  $5 \times 10^{-5}$  M 2-mercaptoethanol (pH 7.3) at 41 °C and 5% CO<sub>2</sub>. Cells were plated in 75 cm<sup>2</sup> tissue flasks (Cellstar, Greiner Bio-one) and cultures were split every 3 days. Cell viability was > 90% by trypan-blue exclusion (Sigma-Aldrich Co.). Prior to stimulation with endotoxin dissolved in Phosphate Buffer Saline, cells were cultured at an initial density of  $2.8 \times 10^6$  cells/flask into 25 cm<sup>2</sup> tissue flasks and kept overnight in the incubator, then stimulated with 0.0 (vehicle treated), 0.1, 1.0, 10.0 µg/ml endotoxin which was isolated from *Salmonella typhimurium*-798 utilizing the aqueous butanol-1 extraction procedure as described by Morrison and Leive 1975 (Morrison and Leive, 1975). Cells were collected at 1, 2, 4, and 8 hours after endotoxin stimulation.

### **RNA Isolation, DNase Treatment and QPCR Experiments**

Total RNA was isolated from pooled samples (3 individual 25 cm<sup>2</sup> flasks per treatment, 3 treatment replicates per each of three treatments performed on different days) using RNAqueous<sup>®</sup> (Ambion, Austin, TX) according to manufacturer's instructions. The

mRNA expression levels of *TLR15*, *IL1B*, *IL6*, *IL10*, *IL8*, and *IFNG* were determined by quantitative real-time RTPCR, using QuantiTect SYBR Green RT-PCR (Qiagen, Waltham, MA). Each RT-PCR reaction was run in triplicate for each sample and consisted of either 50 ng or 75 ng total RNA, 12.5 ml QuantiTect SYBR Green master mix, 0.25 ml QuantiTect RT mix, forward and reverse primers, and RNase-free water for a final volume of 25 ml. The QPCR primer sequences have been previously published (Cheeseman *et al.*, 2007; Cheeseman *et al.*, 2008; Higgs *et al.*, 2006).

The QPCR reactions were performed on an Opticon 2 (MJ Research Inc., Waltham, MA). An initial 50°C step for 30 min was followed by 95°C for 15 min and 40 cycles (94 °C for 15s, 59°C for 30s, and 72°C for 30s, for denaturation, annealing, and extension, respectively) for all PCR amplifications. Gene slopes were determined with serial dilutions differing by 10-fold. A melting curve from 60 to 90 °C with a reading at every 1 °C was also performed for each individual RT-PCR plate. Adjusted cycle threshold (C(t)) values were calculated as follows:

$$40 - [C(t) \text{ sample mean} + (C(t) \text{ 28s median} - C(t) \text{ 28s mean})] * (\text{gene slope}/28\text{s slope})$$

for all genes except *IFNG*. The threshold of 40 cycles was raised to 45 cycles for *IFNG*, because most adjusted cycle numbers were greater than 40. Mean adjusted C(t) values of each triplicate of assays were used in statistical analysis. All RNA samples were DNase treated with DNA-Free (Ambion, Austin, TX) according to manufacturer's instructions before QPCR. The fold changes in mRNA levels were determined as follows:

$\Delta C(T) \text{ non-stimulated} = C(T) \text{ target gene non-stimulated} - C(T) \text{ 28s non-stimulated}$ .  $\Delta C(T) \text{ stimulated} = C(T) \text{ target gene stimulated} - C(T) \text{ 28s stimulated}$ . The fold change in mRNA =  $2^{(\Delta C(T) \text{ non-stimulated} - \Delta C(T) \text{ stimulated})}$

### Statistical Analysis of QPCR Data

The mRNA expression levels for each gene were analyzed with the JMP software (SAS Institute, Cary, NC) ANOVA model. The main fixed effects were time (1, 2, 4, 8 hours) and ST-798 endotoxin dose (0.0, 0.1, 1.0, 10.0 µg/ml) and the interaction of these effects. Multiple comparisons of least squares (LS) means for dose and time effects were determined by Tukey-Kramer honestly significant differences test using JMP statistical software ([SAS Institute, 2005](#)).  $P < 0.05$  was considered as statistically significant.

### Microarray Statistical Analysis

The microarray experiment was conducted using three replications. The first two replications each used one experimental unit and one Affymetrix GeneChip for each of the eight combinations of endotoxin dose (treated vs. control) and time (1, 2, 4, or 8 hours after treatment). The third replication was analyzed with four GeneChips for four endotoxin-treated experimental units measured at 1, 2, 4, and 8 hours after treatment, respectively. Data are deposited in the NCBI GenBank gene expression omnibus (GEO) repository (<http://www.ncbi.nlm.nih.gov/geo/info/linking.html>), series accession number is GSE23881. Data were normalized and expression measures computed using the Robust Multiarray Average (RMA) method (Irizarry *et al.*, 2003). A linear model with fixed effects for

replication, endotoxin dose, time, and interaction between dose and time were fit to the expression data for each gene using the R package limma (Smyth, 2004); Smyth, 2005). As part of each linear model analysis, *P*-values were obtained for the test for dose-by-time interaction, the test for changes over time within endotoxin dose groups, and the test for a dose effect at each time point. The *P*-values for each test were converted to *q*-values for false discovery rate estimation using the method of Nettleton *et al.* (2006) (Nettleton, 2006). The fold changes from microarray data are presented as log base 2.

### **Gene Network Analysis:**

Probe set gene names were downloaded from [www.affymetrix.com](http://www.affymetrix.com). Construction and statistical significance of gene networks were performed by using Ingenuity Pathways Analysis (Ingenuity® Systems, [www.ingenuity.com](http://www.ingenuity.com), henceforth abbreviated as IPA) and by selecting *Gallus gallus* in settings. Statistically significant networks were considered those with a *P* value cut-off of 0.0001. Genes were categorized using IPA. The IPA was also used to identify networks of interacting genes. Genes with *q* values less than 0.05 were entered into IPA.

### **Authors' contributions**

CC carried out the experiments and data analysis for QPCR, gene networks (IPA), participated in the design of the study and drafted the manuscript. SJL, CKT, MJW, DN participated in the design of the study. MJW provided endotoxin. DN performed the statistical analysis of microarray. All authors read and approved the final manuscript.

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**Tables**

Table 1. Effect of endotoxin dose and time on cytokine expression in HD11 macrophages (P values)

<b>Genes</b>	<b>Time</b>	<b>Dose</b>	<b>Interaction</b>
TLR15	0.026	0.002	0.693
IL1B	<0.0001	<0.0001	0.674
IL8	<0.0001	<0.0001	0.539
IFNG	<0.0001	0.376	0.802
IL10	<0.0001	0.429	0.783
IL6	0.014	0.034	0.018

Table 2. Separation of dose effect on HD11 macrophages over time, significance at  $P < 0.05$ 

<b>Genes</b>	<b>0.0</b>	<b>0.1</b>	<b>1.0</b>	<b>10.0</b>
TLR15	x	xy	y	y
IL1B	x	y	z	yz
IL8	x	y	y	y
IFN	x	x	x	x
IL10	x	x	x	x
IL6	x	x	y	xy

x,y,z = doses not sharing a letter are significantly different at  $P < 0.05$ , by Tukey-Kramer Honestly test



Table 3. Effect of endotoxin on cytokine expression at 1, 2, 4 and 8 hours, P values.

<b>Genes</b>	<b>1hps</b>	<b>2hps</b>	<b>4hps</b>	<b>8hps</b>
TLR15	0.027	0.290	0.210	0.136
IL8	0.003	0.027	0.027	0.078
IL1B	0.013	0.004	0.063	0.003
IFNG	0.959	0.412	0.425	0.517
IL6	0.034	0.825	0.002	0.251
IL10	0.336	0.555	0.520	0.946

Table 4. Fold changes [ $\log_2(\text{treated/control})$ ] at 1 hour post-stimulation,  $q$  values from microarray analysis

<b>Gene name</b>	<b>Accession number</b>	<b>fold change <math>\log_2(\text{t/c})</math> at 1 hps</b>	<b><math>q</math>-values</b>
TNFAIP3	XR_026935	2.64	4.29E-07
NFKBIA	NM_001001472	2.29	4.93E-05
MRGPRH	XM_423677	2.29	0.0003
BTG2	XM_418053	2.14	0.0005
IL1B	NM_204524	5.65	0.0009
CCL4	NM_001030360	5.65	0.0009
CD83	XM_418929	3.48	0.009
IL8	NM_205498	2.96	0.01
CH25H	XM_421660	2.46	0.02
TRAF3	XM_421378	1.86	0.03
JUN	NM_001031289	1.62	0.03
IL8	NM_205018	3.03	0.03
TNIP2	NM_001031166	1.74	0.03

Table 5. Effects of endotoxin on genes involved in ubiquitination at 4 hps.

<b>Gene name</b>	<b>Accession number</b>	<b>Fold change log<sub>2</sub>(t/c) at 4 hps</b>	<b><i>q</i>-values</b>
IRAK2	NM_001030605	0.82	0.005
UBE2Q2	XM_413740	0.34	0.045
UBE3C	NM_001030967	0.35	0.049
UBE2A	NM_204865	0.37	0.036
UBE2J1	NM_204763	0.44	0.028
UBE4B	XM_417607	0.73	0.013

## Figures

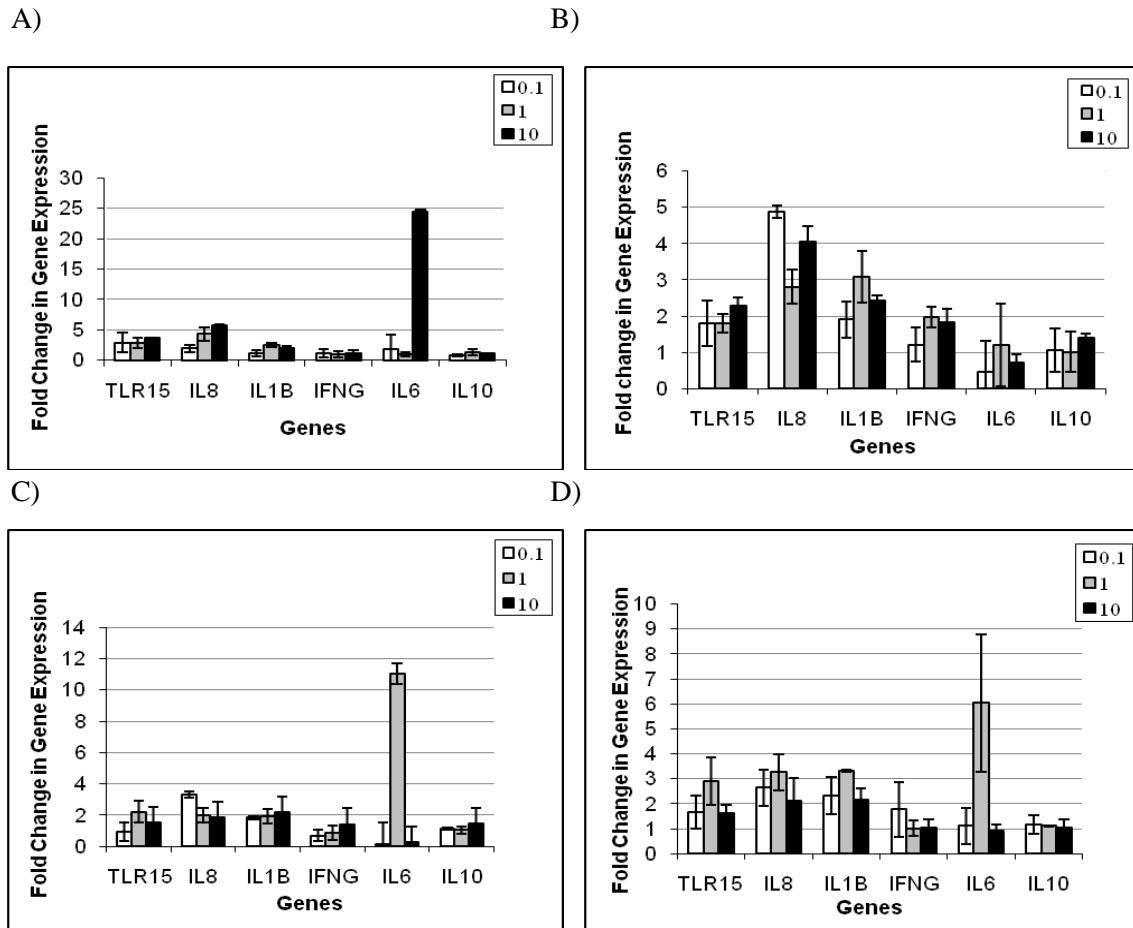
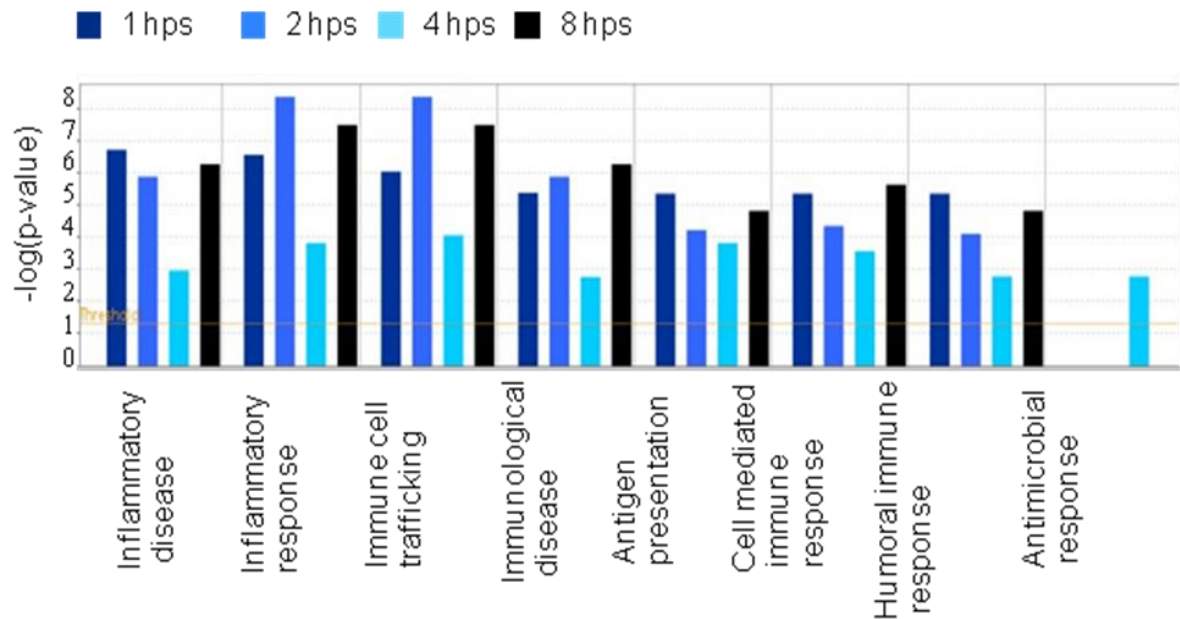


Figure1. Endotoxin stimulation of *TLR15*, *IL8*, *IL1B*, *IFNG*, *IL6* and *IL10* gene expression in HD11 cells. Cells were stimulated for 1, 2, 4 and 8 hours with 0.0, 0.1, 1.0, 10.0 µg/ml endotoxin. Data are shown as the fold change in mRNA levels after treatment compared with nontreated cells by QPCR. A: fold change at 1 h post-stimulation (hps); B: fold change at 2 hps; C: fold change at 4 hps; D: fold change at 8 hps. RNA samples were isolated on 3 different days and the QPCR was carried out in triplicate.



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Fig. 2. Significance levels of different immunological functions of chicken HD11 cells stimulated with endotoxin. Immunological functions at 1, 2, 4, and 8 hours post stimulation. P-values were calculated by Fisher's exact test using IPA. Threshold was set at  $P = 0.05$  and indicated as  $-\log(p\text{-value})$  on the Y-axis. X-axis shows immunological function. Experiments carried out in triplicates.

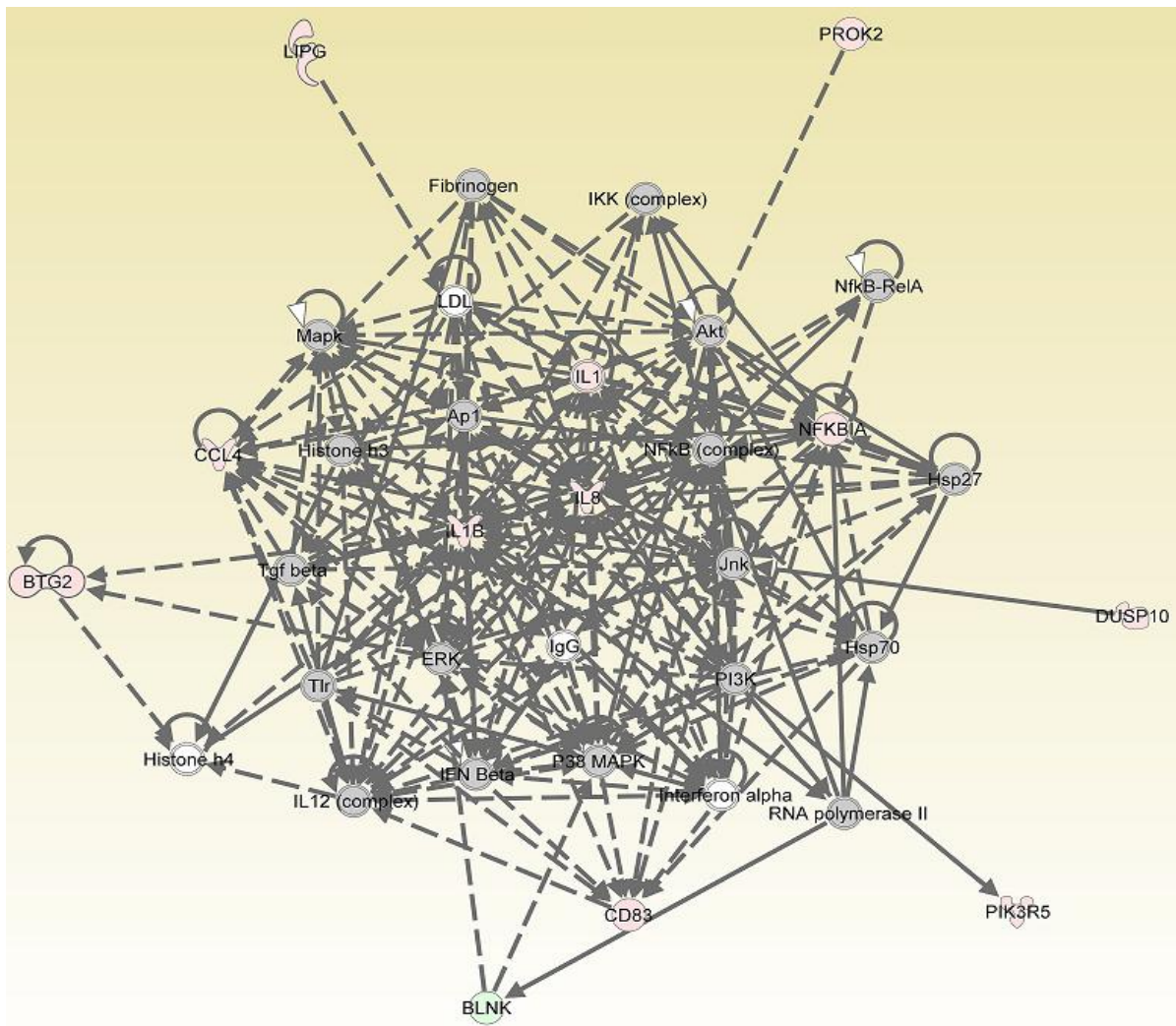


Fig. 3. Gene network analysis of microarray of chicken HD11 cells at 2 hours after endotoxin stimulation. “Cell-to-cell signaling and interaction, hematological system development and function, immune cell trafficking” gene networks at 2 hps. Red color shows up-regulation and green color shows down-regulation (IPA). Grey molecules are not differentially expressed, but are included to illustrate association with significantly up-regulated genes. Experiments were carried out in triplicate.

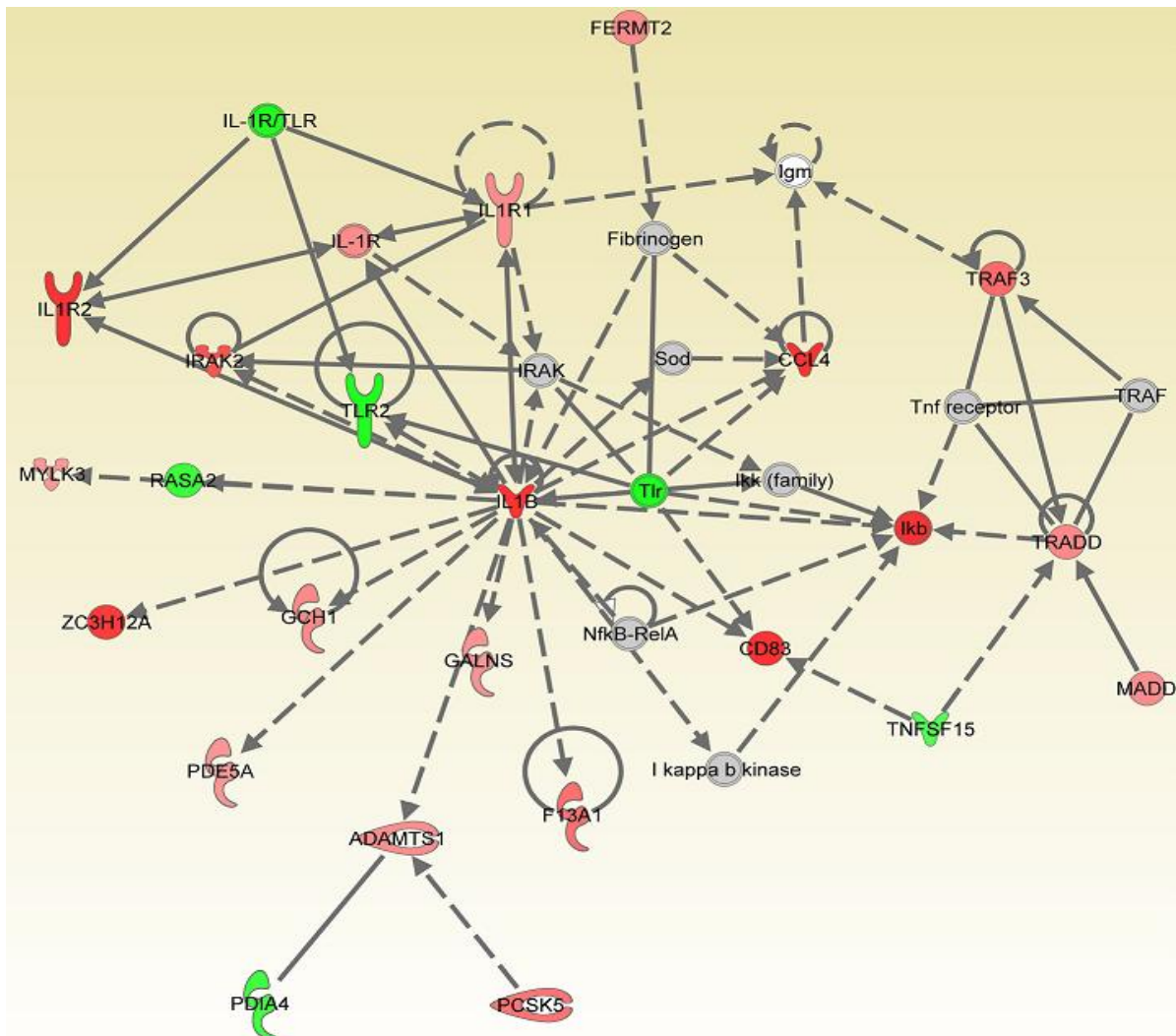


Fig. 4. Gene network analysis of microarray of chicken HD11 cells at 4 hours after endotoxin stimulation. Experiments were carried out in triplicate.

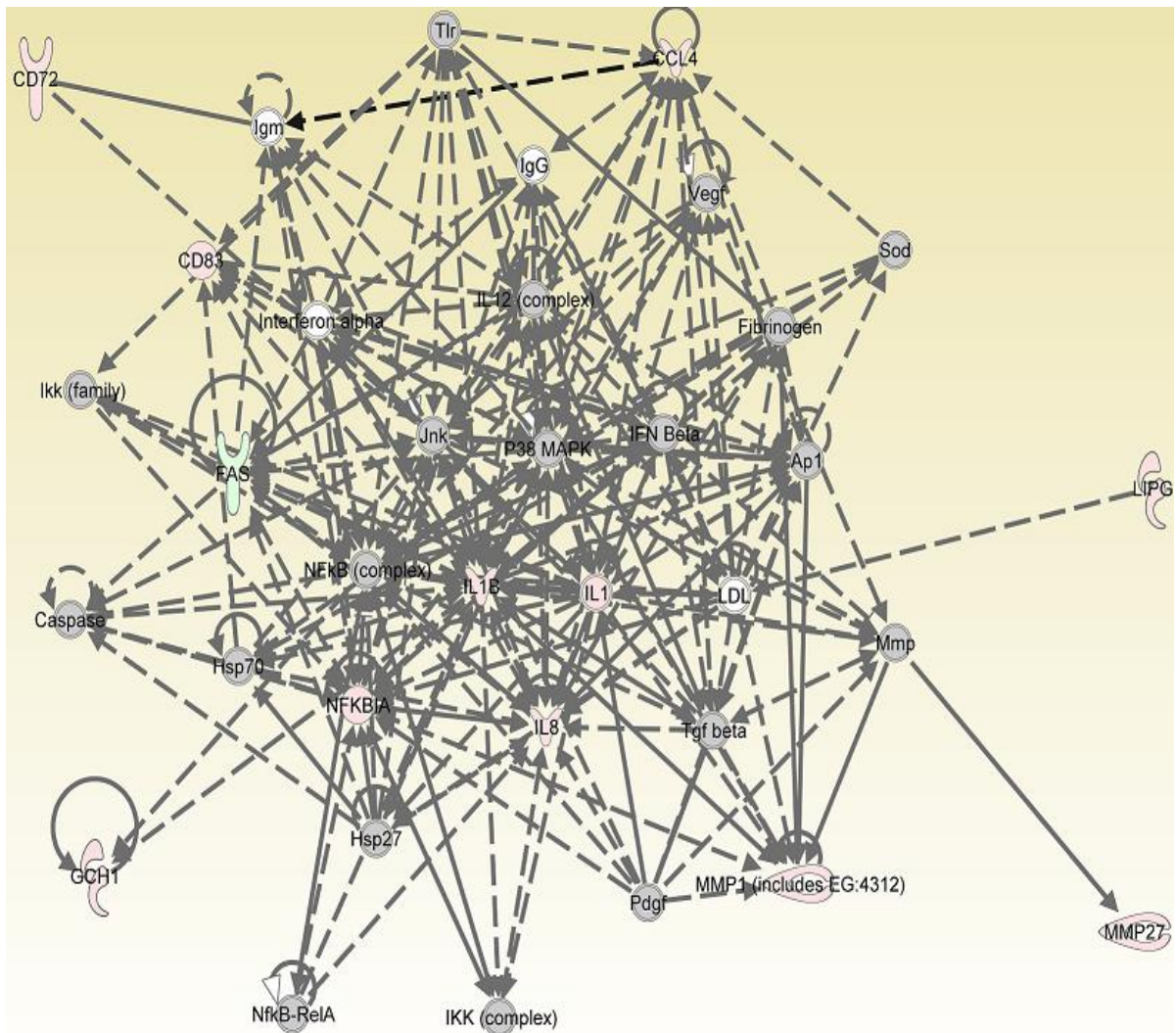


Fig. 5. Gene network analysis of microarray of chicken HD11 cells at 8 hours after endotoxin stimulation. Experiments were carried out in triplicate.



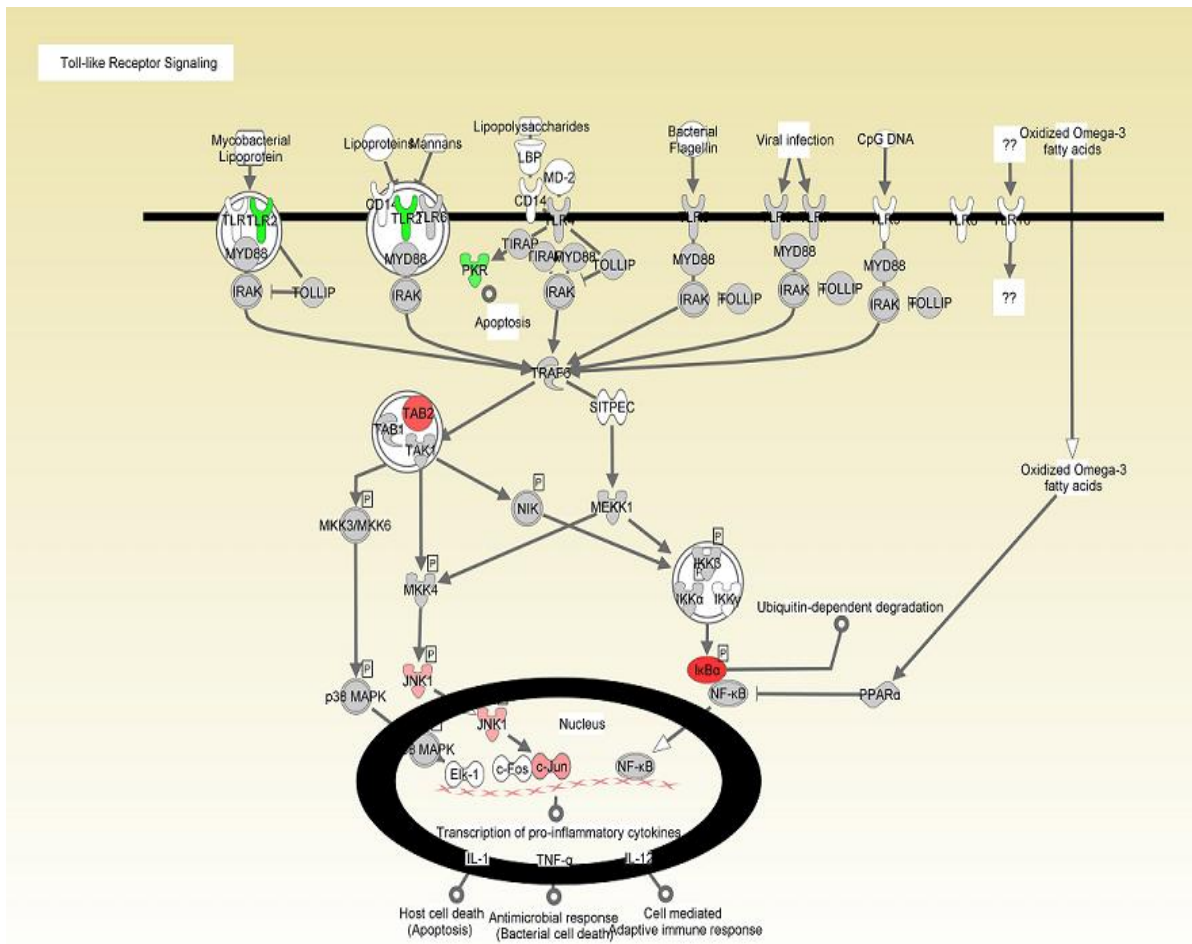


Fig.6. Toll-like Receptor signalling canonical pathway.

Toll-like Receptor signalling canonical pathway attained by IPA obtained from 4 hours post-stimulation data. Up- and down-regulated genes in red and green, respectively. Experiments were carried out in triplicate.

**Additional files****Additional file 1 –**

**Differentially expressed genes clustered by function at each time point.**

Excel file

**Additional file 2 –**

**All genes list; fold changes (stimulated vs non-stimulated) and q values by time for DE genes**

Excel file

**Additional file 3 –**

**Functions of molecules (genes) involved in each gene network, by time.** Interaction with all possible molecules including genes with q values higher than 0.05.

Excel file

## **CHAPTER 4. GENERAL CONCLUSIONS, DISCUSSION, AND FUTURE DIRECTIONS**

### **Overview**

In this dissertation research, we investigated the chicken innate immune response to several pathogen components, utilizing the chicken macrophage cell line HD11 as a model, and mainly focusing on the rapid host response. The following aspects of chicken macrophage response were explored: 1) innate immunity via avian-specific Toll-like receptors (TLRs) in response to specific microorganism associated molecular patterns, 2) mechanism of action of these TLRs in response to nucleic acids, and 3) global expression profiles of chicken macrophages to *Salmonella*-derived endotoxin.

The scope of chapter 2 was to investigate the chicken macrophage host response to pathogen-derived compounds by stimulating cells with CpG-ODNs, synthetic lipoproteins and lipopolysaccharide. The different sets of TLR genes among different species raise interesting questions for comparative immunology. How do the species differently use their distinct TLR receptors to recognize a wide range of pathogen-derived components? How do the different species overcome the absence of specific TLRs and does the presence of additional TLRs make some species more advantageous than others? These differences may be attributed to alterations that occurred over evolutionary time. The current work aimed to gain a better understanding of comparative immunology as we characterize avian-specific

TLRs. We presented evidence of commonality in the mode of action of CpG-ODN responsiveness in mammals and *Aves*.

In chapter 3 we defined the chicken macrophage response to *Salmonella* endotoxin, a Gram negative bacteria outer membrane component. Although response to endotoxins from different sources of bacteria has extensively been studied both in mammals and chickens (De Boever *et al.*, 2009; Foster *et al.*, 2007; Keesstra and van Putten, 2008; Medzhitov *et al.*, 1997; Okamura *et al.*, 2005; Toshchakov *et al.*, 2002), we provided a global expression profiling study in chickens with more comprehensive biological interpretations for the relevant scientific audience.

Proper control of disease-causing pathogens is a major problem for the poultry industry. While conventional pharmaceutical methods are limited to in-feed antibiotics and active vaccination for disease control, increasing public concerns on the use of antibiotics in animal production (Kogut *et al.*, 2010), and failure of current vaccines to completely protect against emerging new strains of pathogens, are becoming a major concerns. Therefore, there is a necessity for better control methods against pathogens. In addition to development of better strategies for disease control, basic research to better understand the host-pathogen interactions at local sites of infection is highly important for production of safe poultry products and public health. Achieving effective control strategies against pathogens can be aided by comprehensive analysis of the basic immunobiology of host-pathogen interactions. The chicken genome sequence is now available (Hillier, 2004) and we have started to understand the genomic complexity both at the DNA and gene expression levels. The

intensity of genomic and molecular data generated from rapid use of new techniques has increased, and many sophisticated methods and algorithms have been developed for comprehensive analysis of these types of data.

The application of genomic technologies for host immune response research is shifting toward the analysis of relevant samples derived from live birds to discover new biomarkers for early detection of diseases. Characteristic patterns of gene expression can globally be measured by microarrays, therefore, gene expression profiling with microarrays is a powerful approach to comprehensively study the transcriptome of early host response to specific stimulants. This approach provides insight into mechanisms governing host response, and also helps to find signatures of genes for a specific type of infection (Li *et al.*, 2010; Lillehoj *et al.*, 2007).

The current kinetic genome-wide analysis in HD11 cells has added to our understanding of gene expression in the *Salmonella typhimurium*-endotoxin stimulated chicken macrophages. More than 1700 of the over 35000 genes were detected to be differentially expressed in the chicken macrophages after 4 hours of endotoxin. This time-dependent microarray study gave us the opportunity to compare and evaluate the chicken host response at different time of exposures. Most differentially expressed genes at all time points were upregulated, rather than downregulated, after endotoxin induction (Fig. 1). Not only did we determine the genes that were significantly differentially expressed after stimulation with endotoxin, but also defined the gene networks involved in immune cell trafficking. The identification of gene networks that are involved in endotoxin response was

informative in describing the importance of exposure time to boost an immune response in macrophages. The complexity of gene networks were directly associated with the number of differentially expressed genes at 1, 2, 4, and 8 hours of exposure. There were very few differentially expressed genes at 1, 2 and 8 hours, which resulted in the formation of poorly refined networks and limited our choice of molecular function in generation of networks. To make a consistent comparison of gene networks at each time point, we chose the same molecular function, which is immune cell trafficking network, in formation of gene networks. According to the present study, 4 hours of endotoxin stimulation is the critical time point for the greatest immune response and the gene sets that are likely to be involved in the immune cell trafficking.

The power of expression profiling utilizing chicken genome microarrays has previously been reported by many groups (Bliss *et al.*, 2005; Lee *et al.*, 2010; Li *et al.*, 2010). However, a limited number of papers have been published on the gene expression profiles of macrophages in a time-course experiment in response to natural bacterial components. Therefore, it is important for the research community in chicken immunity and disease resistance to have access to gene lists that demonstrate expression differences between stimulated and non-stimulated cells to aid understanding of the regulatory mechanisms involved in early host response. The accessibility of comprehensive data sets facilitates the gene annotation procedure and the functional analysis of the uncharacterized genes or gene fragments regardless of the species. The microarray data from the chapter 3 of this

dissertation was therefore deposited into Gene Expression Omnibus (GEO) website for public access.

Although the microarray analysis did not show any TLR induction in response to endotoxin, it presented the novel observation of significant induction of NLRC5 (CARD domain containing, NOD-like receptor family, RCJMB04\_18i2), an intracellular receptor that is currently poorly characterized for functional activity both in mammals and *Aves*. The reason for not detecting any chicken TLR induction might be the dose used in this study and might relate to chickens being more resistant to endotoxic shock. NLRC5, as a novel gene, remains largely uncharacterized even in humans. It would be highly interesting to characterize the functional roles of NLRC5 in chicken innate immunity.

### **siRNA vs shRNA-mediated Gene Silencing**

RNA interference (RNAi) is an evolutionarily conserved process involved in gene expression and in cellular defense by the host against transposable elements and virus genome incorporations (McManus and Sharp, 2002). Although RNAi technology has been widely used in mammalian and plant systems (Ding *et al.*, 2004), publications covering the utilization of siRNAs in avian systems are few. RNAi studies are very common in studies of mammalian systems and the siRNAs are already designed, optimized and commercially available. Therefore, experiments usually produce almost fully silenced genes, whereas siRNAs for avian systems need to be designed, optimized and, to date, there is no study reporting the complete knockdown of siRNA targeted avian genes.

siRNA and shRNA both have advantages and disadvantages from the mechanistic standpoint. Modifications can be made to improve the efficacy and stability of RNAi agents. Chemically synthesized siRNA is easier to modify through chemistry whereas vector based shRNA, which relies on the host machinery for expression, is more difficult to modify. A study using a luciferase expression system compared the potency of siRNA versus shRNA mediated knockdown *in vivo*; the authors found that siRNA and shRNA are equivalent in potency at higher doses; however, on a molar basis, the shRNA was 250 fold more effective than the siRNA (McAnuff *et al.*, 2007). When shRNAs are used to silence gene expression, one has to clone, has to verify the insert, and to determine how much of the shRNA the cells are expressing. ShRNAs are more difficult to transfect and understand the rules for loop sequences but better for stable transfections. In contrast, siRNAs are preferred for screening, transient transfection, and more practical, skips the DICER step which cuts shRNAs into siRNAs (Rao *et al.*, 2009)

Because of the distinct characteristics of cells (for example, adherent vs non-adherent cells), transfection methods should be optimized regardless of the organism. The delivery of siRNAs can be mediated by forward or reverse transfection, which needs to be optimized to increase the efficiency of transfection. In forward transfection, cells are plated and maintained in the incubator overnight, then are transfected on the following day, however, in reverse transfection, cells are plated while transfection is being carried out. In the present dissertation, forward and reverse transfection approaches were both tested on chicken macrophages HD11 cells to determine the optimum conditions. Transfection efficiency was



calculated by the average number of cells that received the fluorescent siRNAs from seven different fields under fluorescent microscope. We concluded that siRNAs were delivered more efficiently by forward transfection than reverse transfection in HD11 cells (Fig. 2). Therefore, forward transfection was employed when siRNAs targeting MyD88 gene were introduced into HD11 cells.

In addition to RNAi delivery methods, concentrations of siRNAs or shRNAs must be optimized due to the potential side effects caused by exogenously introduced siRNAs or shRNAs. These side effects can be defined as follows; 1) endogenous RNAi machinery might compete with exogenously introduced siRNA or shRNAs and stimulate responses to these siRNAs or shRNAs, 2) siRNAs or shRNAs can knockdown irrelevant target mRNAs because of the partial complementarities (Castanotto *et al.*, 2007), or 3) siRNAs or shRNAs may produce serious cellular toxicity and result in cell death, 4) siRNAs or shRNAs can induce interferon response (Judge *et al.*, 2005). Therefore, we also optimized the siRNA dose using fluorescent siRNAs.

The induction of immune cells by siRNAs is a sequence-specific process and sense or antisense strands can individually induce cytokine production as efficiently as duplex siRNA (Marques and Williams, 2005). Synthetic siRNAs designed in nonviral delivery vehicles can be potent inducers of interferons (IFN) and inflammatory cytokines both in vivo in mice and in vitro in human blood (Judge *et al.*, 2005). Thus, the immunostimulatory “side effects” of siRNAs was taken into account when inducing RNAi. To limit the IFN response and other stimulatory effects, we used frequently updated siRNA-design software, to exclude

sequences that are potentially immunostimulatory and the ones that have off-target effects. The outcome of the siRNA-mediated MyD88 gene silencing was 70% reduction in the expression in the present study.

### **Chicken macrophage response to CpG-ODNs**

Despite the apparent absence in chickens of the corresponding mammalian receptor for unmethylated CpG-ODNs (Temperley *et al.*, 2008), responsiveness to CpG-ODNs has been well documented in chickens (Brownlie *et al.*, 2009; He *et al.*, 2003; He *et al.*, 2007; He *et al.*, 2010; Higgs *et al.*, 2006; Nerren *et al.*, 2010). In chapter 2 of the present dissertation, we report that CpG-ODNs regulate the expression of TLR15, TLR2 and TLR21 genes in chicken macrophages HD11 cells. Unlike other TLRs, TLR15 is unique in its responsiveness to multiple stimulators such as PAM3CSK4 (synthetic analogous for lipoprotein), LPS (membrane component from gram negative bacteria) and unmethylated CpG-ODN (Type B and C). There are differences among the studies reporting CpG-ODN responsiveness in avian systems. Recognition of CpG-ODN is sequence-specific; therefore the ODNs containing different motifs can have distinct effects on the same cells. Most of the publications listed above focused on the same CpG-motif containing ODNs, whereas our study utilized multiple different ODN classes.

TLR21, functionally orthologous for human TLR9 (Brownlie *et al.*, 2009), was shown to have a sequence similarity to mouse TLR13 (Keestra *et al.*, 2010). Comparative analysis of protein sequence of chicken TLR21 and mammalian TLR9 demonstrated significant differences in protein configuration of the Leucine Rich Region (LRR) of these

two receptors. TLR21 and TLR9 co-exist in fish; therefore, it would be highly interesting to study the differences between fish TLR21 and fish TLR9 to further characterize the molecular mechanisms of CpG recognition in a comparative immunology viewpoint.

Our studies on CpG response in chicken macrophages provided evidence for the regulatory mechanism of TLRs involved in this process. TLR9 in mammals is known to utilize the MyD88 dependent pathway (Vollmer and Krieg, 2009). This dissertation demonstrates TLR15's ability to respond to several stimulants, which brought the MyD88 dependency of TLR15 to our attention. Utilization of RNAs targeting MyD88 gene added substantial contributions to our knowledge on the CpG signaling action in chickens. We now know that response to CpG via TLR15 and TLR21 operates with the MyD88 dependent pathway in chickens.

### **Summary and future directions**

The main conclusions inferred from the present work are summarized as follows; 1) chicken macrophages, like their mammalian counterparts, can be induced by microorganism- derived components, however, a larger amount is required in *in vitro* studies of chickens, 2) chicken macrophages are able to distinguish and respond to different classes of CpG-ODNs but use a different set of TLRs than mammalian macrophages, 3) response to CpG-ODN operates via MyD88-dependent pathway as does mammalian TLR9, 4) chickens possess a specific TLR15 gene that can rapidly be induced by multiple components from pathogens, and 5) to date, no TLRs have been reported to use both MyD88-dependent and – independent pathways in chickens. Future characterization studies might concentrate on the

other avian-specific TLRs including TLR1La, TLR1Lb, TLR2a and TLR2b, to better understand the regulating mechanisms involved in and participation of MyD88 in cytokine response. Chickens are powerful discovery tools to study immunology, especially innate immunity, in vertebrates. Information from chicken genome-wide expression studies can be used in formation of specific networks at different times, which opens possibilities for production of preventive therapeutic reagents. For example, as mentioned in chapter 3, Tumor Necrosis Factor, alpha-induced protein 3 (TNFAIP3) encoding gene expression was significantly induced at all times after endotoxin stimulation despite the absence of an identified TNFalpha gene in chickens; therefore, it would be highly interesting to study the function of the TNFAIP3 gene in chickens. Our classification by function analysis did not include this gene in the inflammatory response genes category when using Ingenuity Pathway Analysis software (IPA). However, the TNFAIP3 gene is known to be rapidly induced by the tumor necrosis factor (TNF) and has been shown to inhibit NF-kappa B activation (Shembade et al., 2010) as well as TNF-mediated apoptosis (Huang *et al.*, 2010). Knockdown studies for a similar gene in mice demonstrated that this gene is essential for limiting inflammation by terminating TNF-induced NF-kappa B responses (Huang *et al.*, 2010). Therefore, findings from our study could be very critical in developing treatments for animal diseases.

Analysis of the lower vertebrates' immune system using comparative genomics will help in identifying human orthologs involved in governing immune response and, additionally, characterization at a molecular level will help in developing alternative disease

models. Evolutionary and functional studies on the immune response genes will unravel inter-species similarities and differences. Besides these basic research interests, the question remains to what extent immune response changes in macrophages and responses of the immune system to various stimulants could be used as predictors for future development of disease resistance models. The conclusions from these studies might accelerate the application of basic research findings into daily practice to genetically enhance immunity.

Host resistance mechanisms against infections can be enhanced by vaccination and genetics. Therapeutic values of CpG-ODNs and their ability to regulate chicken specific genes expression make CpG-ODNs good candidates for development of DNA vaccines with natural adjuvants as an alternative to widespread use of antibiotics. In addition to the potential of CpG motifs and DNA vaccines, the successful delivery of DNA vaccines has been shown in ovo. However, efficacy needs to be optimized and the cost of production needs to be reduced before their applications in the poultry industry. Another approach to improve the host resistance mechanisms is breeding for disease resistance. Different pathogens have diverse effects on different components of the host innate and adaptive immune defense mechanisms, requiring the studies of host-pathogen interactions with live pathogens in real hosts toward a predictive understanding of the pathogenesis. Our findings lay the groundwork for future genetic research studies to accelerate the decision-making process in genetic selection.

Currently, the distribution of the avian TLRs in the intestinal epithelium is largely unknown. Future studies could investigate the roles of TLRs in immune response and

inflammatory disorders of gut epithelium. Microscopic and functional studies of avian TLRs in the intestinal epithelium could prove to be effective to explore their roles in protection of the host against enteric infections and gut inflammatory diseases.

Additionally, the functional characteristics of avian cytosolic receptors remain to be elucidated. Although expression of NLRC5 and NALP1 genes was shown to be induced by endotoxin and *Campylobacter jejuni*, respectively, nothing is known of the mechanism of action of these receptors. The utilization of gene knock-down methods to understand the specific functions of intracellular receptors would provide new information on the immune parameters of chicken host defense. An in-depth understanding of host resistance should speed the development of specific control strategies using vaccination and genetics, and their application to improve health in poultry and other animals, including humans.

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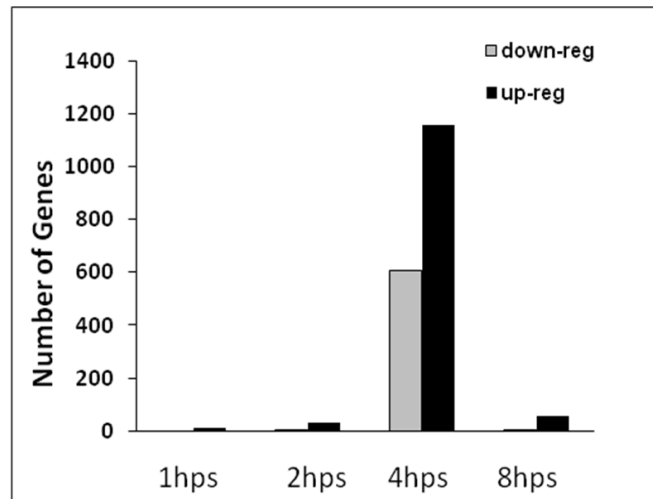
**FIGURES**

Figure 1A. Number of differentially expressed (DE) genes after endotoxin stimulation, by time, compared to non-stimulated cells.

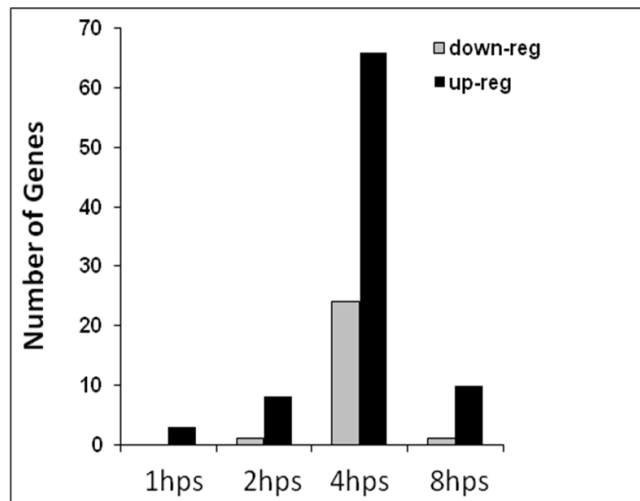


Figure 1B. Number of differentially expressed genes that are involved in inflammatory response, by time.

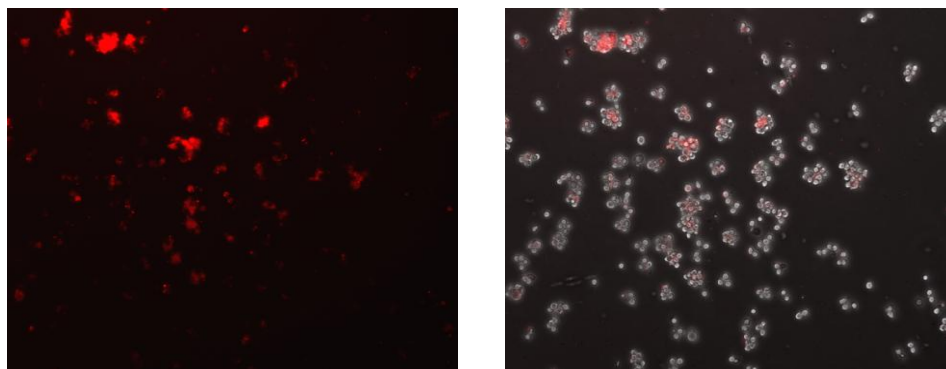


Figure 2A. Forward Transcription with fluorescent siRNAs in HD11 cells

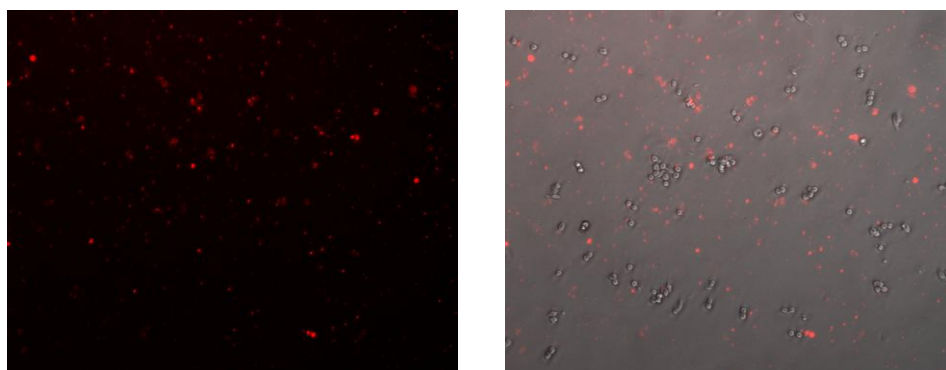


Figure 2B. Reverse Transcription with fluorescent siRNAs in HD11 cells